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The Oxidation of Malic and *meso*Tartaric Acids in Pigeon-liver Extracts

By P. G. SCHOLEFIELD
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(*Received* 23 *August* 1954)

Malic acid is oxidized by malic dehydrogenase and by the malic enzyme, both enzymes requiring the presence of diphosphopyridine nucleotide (DPN) or triphosphopyridine nucleotide (TPN). In the course of experiments on the malic enzyme of pigeon liver, a fraction has been obtained which oxidized sodium DL-malate in the presence of ferricyanide but for which no requirement for DPN, TPN or manganous ions could be shown. The kinetics of this oxidation and its inhibition by other hydroxy acids have been investigated.

Although an effect of *meso*tartaric acid on malic dehydrogenase has not been reported, it is known to affect succinic and lactic dehydrogenase activity in *Escherichia coli* (Quastel & Wooldridge, 1928) and to have some inhibitory action on prostate phosphatase (Anagnostopoulos, 1953). Tartronic (hydroxymalonic) acid, a homologue of malic acid, inhibits malic dehydrogenase (Green, 1936), a concentration of 0·06 M producing 24 % inhibition in pigheart extracts at an unspecified substrate concentration. Tartronic acid also inhibits the lactic dehydrogenase activity of *Esch. coli* but, in contrast to *meso*tartaric acid, has no effect on the succinic dehydrogenase (Quastel & Wooldridge, 1928). It is also an effective inhibitor of lactate oxidation and anaerobic glycolysis but has no effect on pyruvate oxidation in guinea pig brain slices (Jowett & Quastel, 1937).

MATERIALS AND METHODS

Sodium hydrogen DL-*malate.* This was recrystallized 3 times from 80 % (v/v) aqueous ethanol and before use was brought to pH 7·4 with NaOH.

*meso*Tartaric *acid.* A sample from British Drug Houses Ltd., was used without further purification.

DPN and TPN. These were commercial samples obtained from Schwarz Laboratories and Sigma Chemical Co., respectively. Assay of the DPN indicated a purity of 55 %.

Pigeon-liver extract. This was prepared by the method of Mehler, Kornberg, Grisolia & Ochoa (1948). The crude preparation was used, consisting of a dialysed, $(NH_4)_2SO_4$-fractionated extract of an acetone powder.

Standard manometric procedures were adopted, with ferricyanide (Quastel & Wheatley, 1938) and an atmosphere of 93 % N_2–7 % CO_2 at 37°. Substrates and inhibitors were tipped into the Warburg flasks after 3 min. incubation and after a further 3 min. initial readings were recorded.

RESULTS

The oxidation of malate in pigeon-liver extracts

Preliminary experiments confirmed the presence in the pigeon-liver extract of a manganese-requiring TPN-linked malic enzyme dependent upon the presence of a hydrogen-acceptor system such as pyruvate-lactic dehydrogenase (Mehler *et al.* 1948). When attempts were made to substitute ferricyanide for the acceptor system it was found that anaerobic oxidation of malate occurred in the absence of added DPN, TPN or manganese (see Table 1). In the same system no oxidation of *iso*citrate occurred except in the presence of added TPN and none of lactate or β-hydroxybutyrate except in the presence of added DPN. The oxidation of malate, therefore, did not proceed via a soluble pyridine nucleotide in the extract itself.

Since the oxidation of malate could not be due to the presence of malic dehydrogenase, it

Table 1. *The anaerobic oxidation of* DL-*malate and* meso*tartrate in pigeon-liver extract*

All flasks contained 0·5 ml. of 1·3 % sodium bicarbonate, 0·2 ml. of 3 % $K_3Fe(CN)_6$ (higher concentrations gave a precipitate in the presence of added Mn^{2+}), 1·0 ml. of pigeon-liver extract, 0·3 ml. of 0·2 M sodium DL-malate or 0·2 M sodium *meso*tartrate and water to make a final vol. of 3 ml.

Additions			CO_2 evolved in 20 min.	
Mn^{2+} (μatoms)	DPN (mg.)	TPN (mg.)	DL-Malate (μl.)	*meso*Tartrate (μl.)
0·9	0	0·8	123	59
0	0	0·8	127	55
0·9	0	0	127	13
0·9	1·0	0	126	85
0	1·0	0	128	85

was felt that a determination of the Michaelis constant might serve to characterize the enzyme system. The K_m value obtained for DL-malate was $6{\cdot}2 \times 10^{-3}$M or $3{\cdot}1 \times 10^{-3}$M for the L isomer (see Figs. 1 and 2). The value obtained for the pig-heart enzyme was 10^{-2}M and approximately the same value has been reported for both the L-malic dehydrogenase of washed suspensions of *Esch. coli* (Gale & Stephenson, 1939) and the malic enzyme of bacteria (Korkes, Del Campillo & Ochoa, 1950).

The effect of other hydroxy acids on the anaerobic oxidation of DL-*malate in pigeon-liver extracts*

It has been shown (Green, 1936) that 0·06M tartronic acid inhibits malate oxidation by the pig-heart enzyme by 24%. When the effect of tartronic acid on malate oxidation in the pigeon-liver extract was examined and the results analysed by the method of Lineweaver & Burk (1934), it was found that tartronate was a competitive inhibitor of DL-malate oxidation and had $K_I = 9 \times 10^{-5}$M (Fig. 1). From this value it is estimated that tartronic acid is approximately 1000 times as active an inhibitor in the pigeon-liver system as in the pig-heart system.

Furthermore, maleate does not inhibit malate oxidation in pig heart (Green, 1936), but at a concentration of 0·02M it inhibited the oxidation of 0·02M DL-malate in the pigeon-liver system by 50%. Similarly, *iso*citrate (55% at 0·01M) and malonate (30% at 0·02M) both inhibited the oxidation of 0·02M DL-malate under the present conditions. Butyrate, β-hydroxybutyrate and gluconate, however, had no effect on malate oxidation.

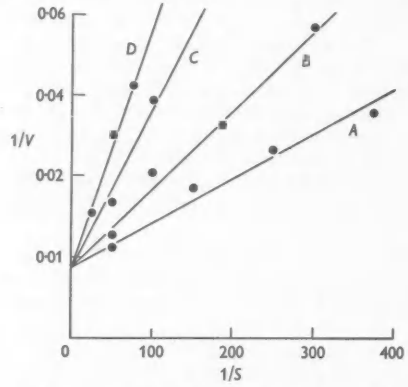

Fig. 1. The competitive inhibition by tartronate of the oxidation of DL-malate in pigeon-liver extracts. Conditions as in Table 1. *A*, no inhibitor; *B*, $6{\cdot}7 \times 10^{-5}$M Na tartronate; *C*, $2{\cdot}2 \times 10^{-4}$M Na tartronate; *D*, $6{\cdot}7 \times 10^{-4}$M Na tartronate; *S* = molar substrate concentration; *V* = velocity, expressed as μl. CO_2 evolved in 32 min.

Citrate was found to be an effective inhibitor, and the results, analysed by the method of Lineweaver & Burk (1934), showed that the inhibition was competitive (Fig. 2) with $K_I = 2{\cdot}65 \times 10^{-3}$M. Sodium D-tartrate and DL-tartrate did not inhibit the anaerobic oxidation of DL-malate, but sodium *meso*-tartrate proved to be a competitive inhibitor with $K_I = 3{\cdot}2 \times 10^{-3}$M. In this connexion, it is interesting to note that the stereoisomers of tartaric acid do not have the same action on prostate phosphatase. This enzyme is strongly inhibited by the D isomer, feebly inhibited by the *meso* isomer and stimulated by the L isomer (Anagnostopoulos, 1953).

The oxidation of meso*tartrate in pigeon-liver extracts*

The particular fraction of pigeon-liver extract under investigation does not oxidize tartronic, citric, *iso*citric, gluconic, maleic, succinic, malonic, butyric, D-tartaric or DL-tartaric acids in the presence or absence of DPN with ferricyanide as hydrogen acceptor. It does oxidize *iso*citric acid in the presence of TPN and shows some activity towards lactate and β-hydroxybutyrate in the presence of DPN, but the rate of oxidation in all cases falls off rapidly owing to accumulation of the products in the absence of fixatives.

However, in the presence of DPN or TPN, *meso*-tartaric acid was oxidized anaerobically by the pigeon liver–ferricyanide system (Table 1). Manganese ions had no effect on the rate of oxidation.

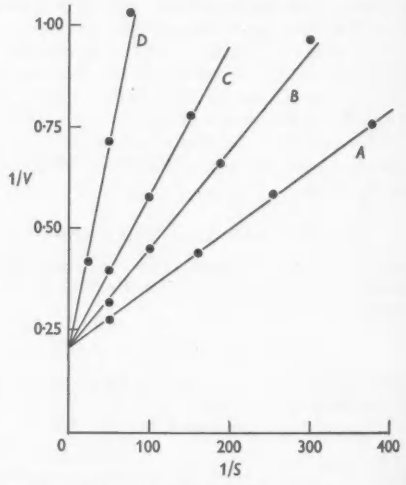

Fig. 2. The competitive inhibition by citrate of the oxidation of DL-malate in pigeon-liver extracts at pH 7·4. Conditions as in Table 1. *A*, no inhibitor; *B*, $1{\cdot}67 \times 10^{-3}$M Na citrate; *C*, 4×10^{-3}M Na citrate; *D*, $1{\cdot}33 \times 10^{-2}$M Na citrate; *S* = molar substrate concentration; *V* = initial velocity, expressed as μl. CO_2 evolved/min.

The K_m value for *meso*tartaric acid in this system is high, $1{\cdot}4 \times 10^{-2}$ M, compared with the K_m value for DL-malate, $6{\cdot}2 \times 10^{-3}$ M. The K_m value found for DPN was approximately 5×10^{-3} M. None of the carboxylic acids tested as potential inhibitors of DL-malate oxidation were found to inhibit *meso*tartaric acid oxidation in pigeon-liver extracts.

Similarly, addition of *meso*tartrate to rat-liver mitochondria resulted in an increased rate of oxygen uptake but much lower activities were found with rat-kidney mitochondria.

DISCUSSION

Sodium DL-malate is oxidized anaerobically by ferricyanide in the presence of pigeon-liver extracts. The oxidation is not catalysed by the malic enzyme since it could be shown that in these extracts TPN is essential for malic enzyme activity, whereas no coenzyme requirement for anaerobic oxidation by ferricyanide could be demonstrated. Further, the marked inhibition of this oxidation by tartronic and maleic acids and the much lower K_m value are not in agreement with the properties of the malic dehydrogenase of pig-heart extracts or resting suspensions of *Esch. coli*. It seems most likely that the responsible enzyme is similar to malic dehydrogenase but with physical constants differing from those found for the enzyme obtained from other sources. A binding of coenzyme by the dehydrogenase similar to that of DPN by glyceraldehyde 3-phosphate dehydrogenase (Velick, Hayes & Harting, 1953) may occur, however, and thus obviate the need for additional coenzyme.

This dehydrogenase is inhibited by *meso*tartaric, tartronic, maleic, malonic, *iso*citric and citric acids, and the inhibition by *meso*tartaric, tartronic and citric acids has been shown to be competitive. The K_I values found are not in agreement with those reported for L-malic dehydrogenase or the malic enzyme of various origins.

*meso*Tartaric acid is oxidized in pigeon-liver extract by a dehydrogenase which requires the presence of either DPN or TPN. With DL-malate or *meso*tartrate oxidation, the products do not seem to inhibit the reaction to a great extent since, in general, the initial velocities are maintained. In contrast, little oxidation of lactate or *iso*citrate could be obtained in the absence of a fixative. The dehydrogenase oxidizing *meso*tartaric acid is not the same as that oxidizing malate since it has a demonstrable DPN or TPN requirement and is not inhibited by any of the compounds shown to inhibit malate oxidation in the same system.

SUMMARY

The kinetics of a characteristic malic dehydrogenase of pigeon-liver extract have been investigated and a DPN-linked dehydrogenase oxidizing *meso*-tartrate in the same system is described.

The present work was performed as part of a programme of investigation of metabolic inhibitors. This programme was supported by a grant to Professor J. H. Quastel, F.R.S., of this Institute, from the National Cancer Institute of Canada.

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Antigenicity of Insulin: Diabetes Induced by Specific Antibodies

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(*Received* 18 *May* 1954)

Lewis (1937) sensitized guinea pigs with ox or pig insulin. Using the Dale technique, he observed contractions of uterine strips from these animals in response to both ox and pig insulin. There was a more persistent response to the preparation of insulin which had been used to sensitize the animal. Bernstein, Kirsner & Turner (1938) have reported anaphylaxis in guinea pigs sensitized and challenged with various insulin preparations. Wasserman & Mirsky (1942) showed that anaphylaxis could be induced in guinea pigs with insulin of a different species than that used for sensitization. Banting, Franks & Gairns (1938) reported the development of resistance to insulin in a non-diabetic schizophrenic patient following a course of insulin shock treatment. Blood serum from this patient, mixed with

insulin and injected into mice, gave a lower incidence of convulsions than did corresponding control mixtures. Lowell (1944) obtained similar evidence for neutralization of insulin with blood sera from two diabetic patients who were resistant to insulin. There is, however, the opinion that insulin is not an antigen. (Haurowitz, 1950; Sherrill & Lawrence, 1950.) In the work which is reported here, it is established that insulin can exercise a true antigenic effect as manifested by anaphylaxis and by the production of neutralizing antibodies.

MATERIALS AND METHODS

Preparation of antigen. Crystalline ox and pig insulin were used for sensitization. The insulin was injected subcutaneously in the form of a stable water-in-oil emulsion, using Freund's adjuvant (Freund & McDermott, 1942). The emulsion was prepared by adding a solution of insulin, 1 mg./ml. (25 units), pH 2·5–3·0 with HCl and containing phenol (0·3%, w/v), to an equal volume of a mixture (9:1, by vol.) of a light paraffin oil (Bayol F, b.p. range 274–380°, Imperial Oil Co.) and mannide mono-oleate (Arlacel A, Atlas Powder Co.). Emulsification was effected by rapid mechanical stirring, using a Waring Blendor or Virtis homogenizer. It is essential that a stable emulsion be obtained, in order to avoid severe hypoglycaemic reactions in the injected animals.

Preparation of crude insulin. Crude insulin extracts were prepared according to the first steps of a method described by Romans (1954). To each 100 g. of minced fresh pancreas were added 250 ml. of 92% (v/v) ethanol and 20 g. of NaCl. The mixture was stirred for 1 hr., during which time the pH was maintained at 5·0–5·2 by the addition of HCl. Solids were then separated by centrifuging and again extracted for 1 hr. with 125 ml. of 70% (v/v) ethanol. The mixture was centrifuged and the two supernatants were combined. Each 100 ml. of the combined supernatants was diluted with 160 ml. of water and shaken in a separating funnel with 25 ml. of CCl_4. The CCl_4 layer was discarded and the aqueous layer dialysed in cellophan sacks against water acidified with HCl to pH 2·5–3·0. The crude insulin extracts of pig, ox, sheep and rabbit at this stage were sufficiently potent for our experimental work. Because of the lower yield (less than 0·5 unit/g. guinea pig pancreas), the dialysed extracts of guinea pig insulin were usually concentrated.

Inoculation of guinea pigs. Each guinea pig was given subcutaneously 1 ml. of antigen into each of two separate areas (a total of 1 mg. of crystalline insulin). This dose was repeated 4 weeks later and at other intervals as needed. Anaphylaxis and neutralizing antibodies can be demonstrated in some inoculated guinea pigs 4 weeks after a single injection of antigen. However, a better response both in respect of anaphylaxis and of neutralizing antibodies is obtained 2 weeks after a second or subsequent injection of antigen.

Bleeding and injecting. Guinea pigs were bled by cardiac puncture and injected intracardially or intravenously. Blood samples were taken from mice by a modification of a method described by Kassel & Levitan (1953). The mouse was held in the left hand of one operator, ventral surface uppermost; the left fore paw was held in the right hand. A piece of cotton gauze attached to a weight of about 50 g. was caught on the incisors and allowed to weigh down the head. A second operator exposed the left external jugular vein using fine scissors and forceps. Blood was drawn with a syringe (0·25 ml.) fitted with a 26-gauge needle; immediately before use the syringe was rinsed with a solution of heparin (1000 units/ml.). The needle was inserted into the vein through the adjoining thoracic muscle in order to avoid tearing the vein. By this technique, four samples (0·1 ml. each) of blood have been taken within 3 hr., without apparent harm to the animal. Injections of 0·5–1·0 ml. of liquid can also conveniently be made into the jugular vein.

Blood-sugar determinations. Samples, obtained as described above, were transferred from the syringe to a 0·10 ml. blood capillary pipette. Blood-sugar estimations were done by the method of Somogyi (1952) with Nelson's (1944) arsenomolybdate reagent.

Acetone bodies. Urine was collected in test tubes from individual mice kept in glass funnels covered with cheese cloth. Acetone bodies were detected in 0·25–1·0 ml. of urine by the method of Legal as modified by Jackson-Taylor (Gradwohl, 1943).

Buffered saline. Phosphate buffer (0·15 M-Na_2HPO_4–HCl, pH 7·4), was combined with an equal volume of 0·9% (w/v) NaCl.

RESULTS

Anaphylaxis in guinea pigs. Guinea pigs were sensitized with crystalline pig or ox insulin according to the method described. Anaphylaxis was readily induced in these animals by intravenous or intracardiac injection of 10–25 units of insulin. For example, of twelve guinea pigs which had received injections of pig insulin as antigen, eight exhibited symptoms of severe anaphylactic shock following an intracardiac injection of 12 units of insulin into each animal. With sensitized animals the symptoms of anaphylaxis including death appeared within 5 min. of the challenging injection, whereas with normal control animals, no symptoms of hypoglycaemic shock were observed within 3 hr. of the injection of corresponding amounts of the same insulin solution, and there were no deaths. It was found that anaphylactic shock could be induced by the injection of pig or ox or sheep insulin into animals sensitized by either pig or ox insulin.

Neutralization of insulin by immune serum from guinea pigs. The neutralizing activity of serum from guinea pigs which had been inoculated with crystalline pig or ox insulin was tested by mouse-convulsion assay procedure. Tests were carried out by injecting starved mice with serum or dilutions of serum mixed with an amount of insulin which, when injected in saline or normal serum, caused convulsions in 95–100% of the mice. The results of a typical test are shown in Table 1 and indicate that 1 ml. of immune serum can neutralize about 1 unit of insulin. The most potent sera which we obtained have shown a neutralizing activity of 4 units of insulin/ml. Sera of the above type, prepared by the

inoculation of crystalline pig or ox insulin, neutralized to the same extent ox insulin and pig insulin. Using the mouse-convulsion technique, it was found that these sera neutralized not only crystalline pig and ox insulin, but also crude pig, ox, sheep and rabbit insulins. No neutralizing activity was ever observed with normal guinea pig, rabbit or sheep sera. The following are typical results. Two groups of twelve mice were injected subcutaneously with mixtures of sheep insulin and serum. In one group the serum was from normal guinea pigs and in the other the serum was obtained from guinea pigs inoculated with crystalline pig insulin. Eleven of the twelve mice in the first group went into convulsions, whereas none of the twelve mice in the second group did. An experiment with rabbit insulin gave similar results. In this experiment normal guinea pig serum was replaced with saline. Six mice which received rabbit insulin and immune serum did not go into convulsions whereas six mice receiving insulin–saline did.

Six batches of guinea pig insulin were prepared. Five of these could not be neutralized by immune serum but one could. This batch was prepared from glands which had been stored in the cold for 6 months. All the other batches were prepared from fresh glands. It must be admitted, nevertheless, that we do not know the conditions necessary for the preparation of the type of guinea pig insulin which can be neutralized by specific serum. The words native and altered will be used to designate non-neutralizable and neutralizable guinea pig insulins respectively.

Neutralization of insulin by immune serum was demonstrated not only by the mouse-convulsion technique but also by changes in blood-sugar levels of mice injected with immune serum and insulin. Some results are shown in Table 2. In the group of mice which had received saline, the injection of pig insulin effected a marked lowering of blood-glucose levels. In the mice which had received immune serum and then pig insulin, glucose levels continued to rise. In the mice which had received immune serum followed by native guinea pig insulin, blood-glucose levels were lower after 2 hr. than after 1 hr. It should be noted that in the group of mice which received native guinea pig insulin, only half as much insulin was injected as in the groups which received pig insulin.

Table 1. *Prevention by immune serum of convulsions due to insulin*

The pooled serum from twelve guinea pigs was used. The animals had been given two injections of crystalline pig insulin with an interval of 4 weeks between injections. Two weeks after the second injection, sera were collected and pooled. The insulin which was used for the test of neutralization was a solution of pig insulin, 1·2 units/ml., pH 2·7 with HCl. Serum, or serum diluted in buffered saline pH 7·4 (1 ml.) was combined with 0·2 ml. of insulin solution; the pH of the mixtures was in the range 6·9–7·4. Separate groups of four mice were used for each mixture. The mice were injected subcutaneously with 0·25 ml. per animal. The number of mice showing convulsions was observed as in the method for insulin assay.

Serum in 1·2 ml. of mixture containing 0·24 unit of insulin (ml.)	No. of mice showing convulsions in a group of 4
1·0	0
0·5	0
0·25	0
0·125	4
0·063	4
0·031	4

Table 2. *Changes of blood-sugar levels in mice following injections of immune serum and insulin*

Mice were divided into three groups of four, and a sample of blood was taken from each mouse. The animals of one group were then each injected intravenously with 0·5 ml. of saline, the animals of the other two groups with 0·5 ml. each of serum from guinea pigs immunized with crystalline pig insulin. One hr. later, blood samples were again taken. Immediately after each mouse was bled it was injected with insulin (0·25 ml.). One hr. after the injection of the insulin, samples of blood were taken for the third time.

	Blood glucose (mg./100 ml.)				
Mouse	Before any injection	First injection	1 hr. after saline or immune serum	Source of insulin injected and dose (units)	1 hr. after insulin injection
1	95	Saline	115	Pig, 0·05	72
2	75		75		14
3	88		97		26
4	110		125		39
5	76	Immune serum	190	Pig, 0·05	260
6	110		215		260
7	78		202		230
8	100		222		260
9	120	Immune serum	235	Native guinea pig, 0·025	155
10	82		195		125
11	81		201		182
12	100		210		129

In other experiments with mice it was shown that immune serum neutralized pig insulin injected 10 min. before the immune serum as indicated by preventing convulsions and reversing the fall in blood-glucose levels. On the other hand, immune serum was ineffective in preventing the continued lowering of blood-glucose levels when native guinea pig insulin was injected 10 min. before the immune serum.

One is justified in ascribing the neutralizing activity of these sera to antibodies which have developed in response to injections of insulin for the following reasons: (1) The guinea pigs which produced the serum were suspectible to anaphylactic shock induced by insulin. (2) The neutralizing activity of the serum was non-dialyzable and was present in the fraction of serum precipitated by 50% and not by 30% saturation with ammonium sulphate. (3) It was possible to release insulin neutralized by serum. This was shown in the following experiment. Serum and insulin were combined at neutral pH, and left overnight in a cold room. The combined serum and insulin was then divided into two equal portions. One portion was acidified to pH 2·3 (3 drops of M-HCl/ml.) and left at room temperature for 1 hr. The acidified portion caused convulsions in all four mice injected. The untreated portion caused no convulsions in the four mice injected.

Diabetes induced in mice by immune guinea pig serum. It is shown in Table 2 that the injection of immune serum into mice was followed by a rise in blood-glucose levels. Results which bear directly on this question are given in Table 3. The glucose concentrations of the immune and normal sera in this experiment were substantially equal, namely 79 and 81 mg./100 ml. respectively. With the immune serum group, the blood-glucose levels at 3·5 and at 21 hr. were markedly elevated; 140 hr. after the final injection of immune serum, blood-glucose levels were comparable to those at zero time.

When injections of immune serum were administered daily for 3 days and tests were carried out on the second, third and fourth days, it was found that blood-glucose levels were elevated as shown in Table 4. There was a suggestion that the injections of immune serum caused a decrease in body weight. The results recorded for mice 12 to 17 were obtained in a later experiment. The pooled urine of mice 12 to 15 on the third day gave a strongly positive test for acetone bodies but the test on the urine from the normal serum group was negative. Tests for the presence of acetone bodies were carried out on the urine of each of seven mice which were given daily injections of immune serum as described in Table 4. On the second day all the tests were negative; on the third day, the tests were positive for three, but negative for four, mice. In this experiment the weights of the mice on the first day were 30, 28, 31, 30, 33, 31 and 30 g. and the losses in weight by the third day were 5, 5, 6, 6, 6, 8 and 6 g. respectively. These weight changes in response to immune serum are in accord with the results in Table 4.

Neutralization of insulin by immune serum from a sheep. A sheep was given a series of subcutaneous injections of crystalline pig insulin in Freund's adjuvant over a period of 3 months without producing demonstrable antibodies, as indicated by the mouse-convulsion technique. After a period of 10 months without treatment, an injection of insulin was again given. Two weeks later the serum of the sheep contained neutralizing antibodies. This serum was found to neutralize insulin of the following species: crystalline pig, crystalline ox, crude rabbit, crude and crystalline sheep, and the crude guinea pig insulin preparation which was neutralized by immune guinea pig serum. The immune sheep serum did not neutralize the type of crude guinea pig insulin which was not neutralized by immune guinea pig serum.

Experiments on the effect of immune sheep serum on blood-glucose levels in mice were undertaken but

Table 3. *Elevation of blood-glucose levels in mice due to injection of immune serum*

Blood samples were taken from each of 6 mice which had been starved for 4 hr. Three of the mice were injected with normal guinea pig serum and 3 with immune serum (neutralizing value 1 unit of insulin/ml.). Injections (0·5 ml.) were given to each mouse at zero time (intravenously) and at 1·5, 3·0 and 4·0 hr. intraperitoneally. Blood samples were again taken at 0·5, 3·5, 21 and 144 hr.

	Blood glucose (mg./100 ml.)				
Mouse	0 hr.	0·5 hr.	3·5 hr.	21 hr.	144 hr.
			Normal serum		
1	65	76	68	92	93
2	71	75	64	98	87
3	70	114	84	121	100
			Immune serum		
4	43	91	167	284	58
5	92	180	285	334	Dead
6	61	98	281	304	51

proved inconclusive. The following experiment throws some light on this problem. Four mice were injected with a mixture of equal volumes of normal and immune guinea pig serum. Four other mice were injected with a mixture of equal volumes of normal sheep serum and immune guinea pig serum. The mice in the first group showed a typical rise in blood-glucose levels; the mice in the second group showed no rise, but rather a slight drop below normal. It may be that this effect of sheep serum was in some way due to antibodies which were haemolytic and agglutinating to mouse erythrocytes and which were present in the normal sheep serum. These antibodies were also present in the immune sheep serum.

Concerning the specificity of various insulins. The general health of the guinea pigs and the sheep which were producing antibodies to insulin was excellent. This observation was consistent with the finding that animals possessing serum of high neutralizing capacity had normal blood-glucose levels. The unimpaired health and normal blood-glucose levels of these animals supports the inference that the antibodies present in the animals did not neutralize endogenous insulin. However, the antibodies did neutralize other insulins injected into the animal. This was shown when twelve immune guinea pigs were found to tolerate an amount of pig insulin which brought about convulsions in all of twelve normal guinea pigs, and when the immune

Table 4. *Effect of repeated injections of immune serum on blood-glucose levels and weights of mice*

On the first day, two intravenous injections of 0·5 ml. and one intraperitoneal injection of 1 ml. were given. On the second and third days, two 1 ml. intraperitoneal injections were given each day. The immune serum used had a neutralizing value of 1 unit/ml.

	Blood glucose (mg./100 ml.)			Body weight (g.)		
Day ...	2	3	4	1	3	4
Mouse			Immune serum			
1	248	—	300	18	14	15
2	88	—	267	18	14	14
3	340	—	250	19	16	15
			Normal serum			
4	127	—	110	20	17	19
5	95	—	125	16	14	16
6	118	—	70	18	18	18
7	96	—	85	17	15	18
			Saline			
8	138	—	120	21	20	22
9	114	—	140	19	20	20
10	167	—	145	22	20	24
11	112	—	105	20	21	21
			Immune serum			
12	390	413	—	—	—	—
13	400	438	—	—	—	—
14	180	434	—	—	—	—
15	400	Dead	—	—	—	—
			Normal serum			
16	104	82	—	—	—	—
17	92	143	—	—	—	—

Table 5. *Activity of various insulins in the presence of immune guinea pig and sheep sera*

Both guinea pig and sheep sera were prepared using pig insulin as antigen. The criteria for neutralization were prevention of convulsions and effect on blood-glucose levels. Native guinea pig insulin refers to the preparations which were not neutralized by immune guinea pig serum; altered guinea pig insulin refers to the preparation which was neutralized. Neutralized indicated by +, not neutralized by −.

Insulin											
Pig		Ox		Sheep			Guinea pig				
Cryst.	Crude	Cryst.	Crude	Cryst.	Crude	Endogenous	Native	Altered	Endogenous	Mouse, endogenous	Rabbit, crude
					Immune guinea pig serum						
+	+	+	+	+	+	.	−	+	−	+	+
					Immune sheep serum						
+	.	+	.	+	+	−	−	+	.	.	+

sheep showed no change in blood glucose level following an injection of a large dose of pig insulin (275 units). When crystalline sheep insulin (20 units) was injected subcutaneously into the immune sheep there was again no alteration in glucose level. This result indicates that the prepared sheep insulin did not revert to endogenous insulin after injection. The summary of the results bearing on the specificity of various insulins given in Table 5 indicates that endogenous guinea pig insulin, and native guinea pig insulin are specifically different from endogenous mouse, altered guinea pig and prepared pig, ox, sheep and rabbit insulin. Endogenous sheep insulin is specifically different from crystalline and crude sheep, crystalline pig and ox, crude rabbit and altered guinea pig insulin.

DISCUSSION

The very fact that pig insulin when used as an antigen can induce neutralizing activity for pig insulin in guinea pig serum justifies the conclusion that insulin and not associated impurities is the effective antigen. This is supported by the fact that insulins of species different from that used as the antigen were also neutralized. Results on anaphylaxis were consistent with the preceding conclusion. However, since crystalline insulin may contain appreciable amounts of associated material (Harfenist & Craig, 1952) it is possible, though highly improbable, that anaphylaxis was not due to insulin.

Guinea pig and the sheep insulins have been prepared which were immunologically different from corresponding endogenous insulins. These altered insulins were neutralized by homologous antisera, whereas the endogenous insulins were not neutralized. Certain preparations of guinea pig insulin were like endogenous guinea pig insulin, in that they were not neutralized by homologous immune serum.

Some consideration was given to the question whether altered insulin could revert to endogenous insulin after injection into the species from which it was derived. It is probable that no such change occurs, since there was no effect on blood-glucose level when altered sheep insulin was injected into an immune sheep. In contrast with this finding, native guinea pig insulin was able to lower blood-glucose levels in spite of the presence of excess guinea pig immune serum.

It is rather striking that guinea pig immune serum, prepared by injecting crystalline pig insulin, neutralized not only a series of prepared insulins including pig, ox, sheep, rabbit and altered guinea pig insulin, but also endogenous mouse insulin. Endogenous mouse insulin, therefore, is immunologically distinct from endogenous guinea pig insulin, and it is probable that endogenous insulins in general are species specific. Endogenous sheep insulin is specifically different from prepared ox, pig, sheep, rabbit and altered guinea pig insulin. Since native guinea pig insulin was not neutralized by immune sheep serum, no conclusion can be drawn regarding the immunological relationship of native guinea pig and endogenous sheep insulins.

It has been suggested by Lerman (1944) that resistance to insulin in human diabetics is due to specific antibodies and that human insulin would be desirable for the control of such diabetics. However, since the specificity of an insulin preparation can be different from that of its endogenous insulin, for example guinea pig or sheep insulin, it is probable that the effectiveness of a preparation of human insulin for resistant diabetics would depend on whether it was the endogenous type, like native guinea pig insulin, or the altered type.

Some consideration was given to the way in which immune serum rendered mice diabetic. It is most probable that immune guinea pig serum was effective against mouse insulin alone and not against any other component of the mouse, even though other antibodies than anti-insulin may have been developed in the guinea pig in response to injections of crystalline pig insulin. It follows then that diabetes in mice, as indicated by abnormally high blood-glucose levels, loss of weight, and acetone bodies in the urine, resulted from a simple lack of insulin, preceded by no other bodily derangement.

These considerations do not imply that diabetes in humans is caused by an immunity mechanism. It is most unlikely that immunization with endogenous insulin ever takes place. There is of course acquired resistance to insulin in diabetics which in some cases is due to antibodies to heterologous insulin. Fortunately this phenomenon is relatively rare. Smelo (1948) found that a total of fifty-four cases of resistant diabetics had been reported in the English literature. It would appear then, that insulin is a poor antigen in diabetic humans.

Insulin is also a poor antigen in rabbits. Of thirty rabbits repeatedly injected with insulin mixed with various adjuvants, not one produced antibodies demonstrable by the mouse-convulsion technique. On the other hand, little difficulty was experienced in producing immune serum in guinea pigs.

Attempts to demonstrate *in vitro* flocculation in mixtures of insulin and immune serum were unsuccessful.

SUMMARY

1. Intravenous injection of pig or ox or sheep insulin, caused anaphylactic shock in guinea pigs sensitized with crystalline pig or ox insulin.

2. Neutralizing antibodies for insulin were induced in guinea pigs and in sheep by injections

of insulin in Freund's adjuvant. Animals producing antibodies were resistant to insulin. Serum from these animals neutralized insulin, as shown by inoculation into normal mice. This neutralization took place whether the insulin and serum were mixed together before injection or were injected separately at different times.

3. Endogenous guinea pig and sheep insulins were not neutralized by homologous antibodies.

4. Two types of guinea pig insulin have been prepared which were immunologically distinct. One type, classed as altered, was neutralized by immune guinea pig serum. The other, like endogenous guinea pig insulin, was not neutralized. The sheep insulins tested, which included one commercial preparation, were neutralized by immune sheep serum and therefore may be classed as altered.

5. This study indicates that endogenous guinea pig insulin is immunologically distinct from altered guinea pig insulin, from endogenous mouse insulin, and from preparations of pig, ox, sheep and rabbit insulin; and that endogenous sheep insulin is immunologically distinct from altered guinea pig insulin and from preparations of pig, ox, sheep and rabbit insulin.

6. Mice were rendered diabetic by injection of guinea pig anti-insulin serum. These diabetic mice showed abnormally high blood-glucose levels, loss of weight, and acetone bodies in the urine during the period of administration of serum.

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Arsenolysis and Phosphorolysis of Citrulline in Mammalian Liver

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It has been shown by Knivett (1952*a*, *b*, 1953*a*, *b*, 1954*a*, *b*, *c*), Slade (1953), Slade, Doughty & Slamp (1954), Oginsky & Gehrig (1952, 1953) and Korzenovsky & Werkman (1953, 1954) that (*a*) citrulline can react in *Streptococcus faecalis* and other organisms according to the reaction

$$\text{citrulline} + \text{ADP} + \text{phosphate} \rightarrow \text{ornithine} + NH_3 + CO_2 + \text{ATP}, \quad (1)$$

where ADP and ATP represent adenosine diphosphate and adenosine triphosphate, respectively; (*b*) citrulline can decompose in the presence of arsenate (by arsenolysis) according to the reaction

$$\text{citrulline} + H_2O \xrightarrow{\text{arsenate}} \text{ornithine} + NH_3 + CO_2, \quad (2)$$

no addition of adenosine phosphate or organic phosphate being required in this case; (*c*) citrulline can be split in the presence of phosphate (by phosphorolysis) according to the reaction

$$\text{citrulline} + H_2O \xrightarrow{\text{phosphate}} \text{ornithine} + NH_3 + CO_2, \quad (3)$$

provided that the ornithine formed is removed, e.g. by addition of ornithine decarboxylase.

The present experiments are concerned with analogous reactions in animal tissues. They show that reactions (2) and (3) readily occur in mammalian liver. They are absent from avian liver. The evidence suggests that the reactions are connected with the enzyme system responsible in the intact liver for the synthesis of citrulline from ornithine, CO_2 and ammonia.

MATERIALS AND METHODS

Adenosine triphosphate was prepared according to LePage (1949) and contained 72·5% ATP, 13·5% ADP, and 14% inorganic phosphate. In some experiments a commercial preparation, referred to in the text as ATP–ADP mixture, was used; it contained 34·6% ATP, 30·5% ADP, 7·2% AMP, 11·3% inorganic phosphate and 16·4% unknown organic phosphate. The data are based on chromatographic analysis (Eggleston & Hems, 1952; Krebs & Hems, 1953) and are expressed as percentage of total phosphate.

Arsenate buffers were made by mixing M-Na_2HAsO_4, N-HCl and water. The approximate pH was calculated, the pK of the second ionization constant being taken as 6·7. The actual pH was measured in many cases with a glass electrode after dilution. Phosphate buffers consisted of mixtures of 0·5M-NaH_2PO_4 and 0·5M-Na_2HPO_4. Succinate buffers were mixtures of M succinic acid and 2N-NaOH.

N-Acetyl-L-glutamic acid was prepared according to Nicolet (1930). Carbamyl derivatives of amino acids were prepared in this laboratory by Dr W. Bartley by the following method which has the advantage over other procedures of avoiding prolonged heating and risk of racemization.

The L-amino acid or amide was dissolved in the minimal amount of water and to this was added slightly more than one equivalent of nitro-urea, prepared by the method of Davis & Blanchard (1929). With stirring and heating to 70°, a measured quantity of dry $Ba(OH)_2$ was added to bring the pH to 8–8·5 and was maintained for 30–60 min. Barium was precipitated by gradually adding the calculated amount of H_2SO_4. After cooling and centrifuging, the supernatant solution was concentrated *in vacuo*, adjusted to pH 5·5–6 with NaOH, and treated with excess basic lead acetate, the pH being maintained by the addition of acetic acid. After standing overnight the lead salt was separated and suspended in warm water, and the lead precipitated with H_2S. The clear filtrate was concentrated *in vacuo* until crystals separated. Recrystallization was from water or from ethanol–ether mixtures.

Liver suspensions. Two types of liver preparations were used. Fresh liver suspensions were prepared by homogenizing or blending ice-chilled liver with four parts of water or a saline medium containing 9 parts 0·9% (w/v) KCl and 1 part 0·1M-$MgCl_2$. The stainless-steel homogenizer of the Potter–Elvehjem type and a Waring Blendor, both chilled, were used. The suspensions were stored at −15°. To prepare acetone-dried liver, ice-chilled tissue was disintegrated in a Waring Blendor with 10 vol. acetone at −10°. After filtration on a filter funnel the liver was dried *in vacuo* over H_2SO_4 and stored at −15°. In some cases the acetone treatment was repeated two or three times. The dried material was shaken with 10 or 25 parts of water, or the KCl–$MgCl_2$ solution described above, for 2 hr. at 4° and then centrifuged. The supernatant was stored at −15°. In some experiments it was dialysed against large volumes of ice water for 4–16 hr.

Ornithine decarboxylase. Escherichia coli (E:C:4) (Nat. Coll. Ind. Bact., 8571) was grown on a medium containing 3% enzymic casein digest (Pronutrin), 1% glucose, 0·5% 'Difco' yeast extract, 2·5% agar and 0·1% L-ornithine (all w/v). After incubation for 16–20 hr. at 37°, cells were washed three times in water and then suspended in 0·1M-KCl or water. On storage at 2° the ornithine decarboxylase activity was maintained for several weeks.

Manometric measurement of CO_2 evolution from citrulline. Conical Warburg flasks with one or two side arms were used, a second side arm containing 0·5 ml. 10% (v/v) H_2SO_4. The acid was added at the end of the incubation to release bound CO_2. The total volume of reagents in the flasks was between 2 and 4 ml. The gas space contained N_2, the centre well yellow phosphorus. Citrulline was usually added from the side arm after thermal equilibration. The temperature of incubation was 40°.

Paper chromatography. To identify ornithine as a breakdown product of citrulline the incubated material was deproteinized with about 0·15 vol. of 30% (w/v) trichloroacetic acid (TCA). Of the filtrate 20–50 µl. were dried on to chromatogram papers in a 1 cm. square area with a current of warm air. The papers were developed with a formic acid–*iso*propyl ether solvent of Hanes & Isherwood (1949) for 4 hr. with ascending flow, or alternatively by descending flow, for 16–18 hr., with a mixture of 4 parts liquid phenol and 1 part NH_4OH, sp.gr. 0·880, or phenol saturated with water at 20°. After drying in a current of warm air, the papers were sprayed with 0·4% (w/v) ninhydrin in water-saturated *n*-butanol, and then heated 15 min. at 60°. L-Citrulline and L-ornithine reference spots were run simultaneously.

To test for the presence of carbamyl derivatives of amino acids, a spray containing 4% (w/v) *p*-dimethylaminobenzaldehyde in N-HCl was used (Phillips, 1954). Yellow spots appeared on heating the paper when these compounds were present.

RESULTS

Effect of phosphate and arsenate on CO_2 production from citrulline

When citrulline was added to rat-liver preparations, together with ATP or ADP and inorganic phosphate, no appreciable evolution of CO_2 took place under conditions where bacterial extracts cause a rapid decomposition of citrulline. A very small increase in CO_2 production which just exceeds the limit of error was observed when the phosphate and adenosine phosphate concentrations were 0·01 M, and definitely measurable rates of CO_2 evolution were observed when higher concentrations of phosphate were added (0·025–0·25 M). This is shown in Table 1. The addition of ATP or ADP did not affect the rate of CO_2 evolution, although adenosine phosphates were fairly stable in extracts of acetone-dried liver.

Table 1. *The effect of phosphate concentration on CO_2 production from citrulline*

Guinea pig liver homogenate, 1 in 20 final dilution; 0·05M L-citrulline; pH 6·5; total vol., 2 ml.; 40°; N_2.

Additions	CO_2 formed in 60 min. (µl.)
None	6
Phosphate, 0·025M	18·5
Phosphate, 0·05M	29
Phosphate, 0·125M	35
Phosphate, 0·25M	44
Arsenate, 0·25M	386
NaCl, 0·25M	3

In contrast with phosphate, arsenate was found to accelerate greatly the rate of decomposition of citrulline, both in fresh liver suspensions and in acetone-dried liver extracts (Tables 1 and 2). The stimulating effect of arsenate was diminished by the addition of phosphate. A plot according to Lineweaver & Burk (1934) indicates that the inhibition is competitive.

The formation of ornithine was established by paper chromatography, the formation of ammonia by Nessler's reagent.

Enzymic characteristics of arsenolysis

The pH optimum of the arsenolysis was found to be at about pH 6·7. The optimum is fairly sharp, the activities at pH 6·3 and 7·1 being 72–75% of the maximal activity under the test conditions (Table 3). The rate of arsenolysis increased with the citrulline concentration up to 0·1 M citrulline (Table 4). The Michaelis constant as calculated from Table 4 was about 0·014 M. It varied with the experimental conditions between 0·01 and 0·05, being higher at the lower pH values. The time course of the arsenolysis indicated that the reaction did not go to completion even when low concentrations of substrates with relatively large amounts of enzyme were used (Table 5). The rate of CO_2 evolution rapidly decreased, and after about 90% of the expected amount had been liberated the gas evolution became almost nil.

Storage of fresh liver suspensions or extracts of acetone-dried liver at $-15°$ for 3 months did not reduce the enzyme activity. Extracts made with distilled water were as active as those containing KCl and $MgCl_2$. Prolonged dialysis (16 hr. at 0°) against tap water or distilled water, or addition of boiled liver extract had no effect on the rate of arsenolysis and in none of the conditions tested could arsenate be effectively replaced by phosphate and ADP. The relative reaction rates in the presence of arsenate and phosphate remained of the order shown in Table 1.

Table 2. *The effect of arsenate concentration on CO_2 production from citrulline*

Expt. 1. Aqueous extract of acetone-dried guinea pig liver, 0·5 ml.; 0·025 M L-citrulline; pH 6·7; total volume 2 ml. Expt. 2. Rat-liver homogenate, 1 in 20 final dilution; 0·05 M L-citrulline; pH 6·3; total vol., 2 ml. Buffer concentrations are final; 40°; N_2.

Expt. no.	Arsenate (M)	Phosphate (M)	CO_2 formed in 60 min. (μl.)
1	0	0	0
	0·0625	0	132
	0·25	0	175
2	0	0·02	6
	0·25	0	409
	0·25	0·02	268
	0·125	0	389
	0·125	0·02	233
	0·0625	0	314
	0·0625	0·02	151

Table 3. *The effect of* pH *on the arsenolysis of citrulline*

Rat-liver homogenate, 1 in 20 final dilution; 0·05 M L-citrulline; 0·25 M arsenate buffer; total vol., 2 ml.; 40°; N_2.

	CO_2 formed in 60 min. (μl.)		
pH	Free	Bound	Total
5·59	8	0	8
6·02	49	0	49
6·27	216	10	226
6·70	260	54	314
7·14	148	88	236
7·63	59	104	163

Table 4. *Effect of citrulline concentration on the rate of arsenolysis of citrulline*

Each flask contained 0·5 ml. dialysed guinea pig liver homogenate (1 part liver, 4 parts water); 0·5 ml. 0·5 M arsenate buffer, pH 6·7; 0·05–1·0 ml. 0·5 M DL-citrulline. Total vol., 2 ml.; N_2. Readings began after 10 min. equilibration. Free CO_2 was about 80% of total CO_2.

	Final citrulline concn. (M)					
	0·125	0·10	0·05	0·025	0·0125	0·00625
μl. CO_2 (free) after 10 min.	70	70	56·5	47·5	35	22·5
μl. CO_2 (free) after 60 min.	288	283	232	192	140	91·5

Table 5. *Time course of arsenolysis of citrulline*

Guinea pig liver homogenate, 1:13; 0·25 M arsenate buffer, pH 6·7; 0·0031 M L-citrulline. Total vol., 4 ml.; N_2.

Time (min.)	Total CO_2 formed (μl.)	% of CO_2 expected for complete hydrolysis
30	110	42
60	172	62
90	213	76
120	237	85
150	249	89

Table 6. *The relative arsenolytic activities of fractions obtained from sheep-liver mitochondria*

For details see text. Enzymic assay carried out at 40° in N_2. Q_{CO_2} = μl. CO_2 liberated/mg. dry wt. of enzyme/hr.

Enzyme fraction	Whether dialysed	Q_{CO_2}
Whole suspension (in water) of washed mitochondria	No	10·5
Lysed mitochondria, sediment	No	9·8
Lysed mitochondria, supernatant	No	33·1
Lysed mitochondria, supernatant	Yes	57·8
Ppt. from 20% saturation of supernatant with $(NH_4)_2SO_4$	Yes	13·3
Ppt. from 40% saturation of supernatant with $(NH_4)_2SO_4$	Yes	16·1
Ppt. from 60% saturation of supernatant with $(NH_4)_2SO_4$	Yes	156·8
Ppt. from 80% saturation of supernatant with $(NH_4)_2SO_4$	Yes	40·6
Ppt. from final acidification with TCA to pH 2·0	Yes	6·2

Table 7. *Inhibition of the arsenolysis of citrulline by ornithine*

Guinea pig liver homogenate, 1 in 20 final dilution; 0·0375M L-citrulline in Expt. 1, 0·025M in Expt. 2; 0·25M arsenate buffer in Expt. 1, 0·125M in Expt. 2; pH 6·7; total vol., 2 ml.; 40°; N_2.

Expt. no.	Additions	CO_2 liberated in 60 min. (μl.)
1	None	287
	L-Ornithine, 0·02M	133
	L-Ornithine, 0·02M, NH_4Cl, 0·02M	131
	NH_4Cl, 0·02M	276
2	None	316
	L-Ornithine, 0·05M	62
	L-Ornithine, 0·0125M	167
	L-Ornithine, 0·00625M	226

Purification of the enzyme

Liver mitochondria prepared by the method of Bartley & Davies (1954) were found to contain the enzyme, whilst the supernatant was inactive. After treatment of the washed mitochondria with 5 vol. of water, alternate freezing at −15° and thawing at 2° (twice) and centrifuging, most of the enzyme was in the clear supernatant. Treatment with ammonium sulphate led to precipitation when the salt concentration reached 60–80% saturation. The relative enzymic activity of various fractions is shown in Table 6. For assaying, the material was dissolved in 0·125M arsenate buffer, pH 6·7, containing 0·1M DL-citrulline. It will be seen that the enrichment in the most active samples was about 15-fold. There was a loss of about 50% of the total activity during the ammonium sulphate fractionation.

Effects of various substances on the arsenolysis and phosphorolysis of citrulline

The following substances were found to be without effect on the arsenolysis of citrulline (pH 6·7, 0·25M arsenate and 0·025M L-citrulline): D-citrulline (0·05M), L-glutamate (0·01M), carbamyl-L-glutamate (0·05M), succinate (0·02M), *sec.*-octanol (50 mg.), cetyltrimethylammonium bromide (0·1%, w/v), hydroxylamine (0·01M), $BeCl_2$ (0·001M), $FeSO_4$ (0·01M) and KCN (0·001M). Iodoacetate (0·01M) and mercaptoacetate (0·01M) caused small inhibitions (about 10%); *p*-chloromercuribenzoate (0·01M) inhibited almost completely. 0·05 and 0·10M BAL (2:3-dimercaptopropanol) were also inhibitory.

A specific inhibition is caused by one of the end products of the reaction, L-ornithine. The magnitude of the effect is shown in Table 7; at 0·0125M the inhibition was about 50%. The other end products, ammonium ions and CO_2, had no effect. The inhibition by ornithine provides an explanation for the incomplete fission of citrulline already mentioned.

Attempts to replace arsenate by other agents were unsuccessful. Among those tried were 2:4-dinitrophenol (10^{-3}–10^{-4}M) and NaF (0·05M). Attempts were also made to increase the rate of CO_2 production from citrulline in the presence of phosphate and ADP and absence of arsenate. The following substances were tested and none of them was found to be effective: Ca^{2+} or Mg^{2+} (0·0025–0·02M), ethylenediaminetetra-acetate (0·005–0·01M), L-lysine (0·01M), L-histidine (0·01M), L-cysteine (0·005–0·02M), glutathione (0·003M), L-ascorbic acid (0·01M), BAL (0·0025–0·05M), $BeCl_2$ (0·001M) Mn^{2+} (0·005M), Fe^{2+} (0·01M), NH_4Cl (0·05M), cetyltrimethylammonium bromide (0·1%, w/v), coenzyme A (~10^{-4}M), flavin-adenine dinucleotide (~10^{-5}M), flavin mononucleotide (~10^{-5}M), L-glutamate (0·05M), L-glutamine (0·05M), L-aspartate (0·05M), L-asparagine (0·05M), carbamyl-L-glutamate (0·05M), carbamyl-L-glutamine (0·05M), carbamyl-L-asparagine (0·05M), α-oxoglutarate (0·05M), pyruvate (0·05M), oxaloacetate (0·05M) and glucose (0·05%, w/v). Various crude enzyme preparations were added to the phosphate-buffered system as it was thought that the slow rate of reaction might be due to the absence or low concentration of some factor in the liver enzyme required to split a hypothetical citrulline–phosphate postulated by previous authors. The préparations which were tried and were without effect included: intestinal phos-

phatase, brewer's yeast extract (Lebedev juice), rat-kidney homogenate, aqueous extract of soya bean, and cobra venom.

Nature of the inhibition by ornithine

The fact that ornithine only among the three end-products is inhibitory suggests that the inhibition is not due to a mass-action effect but to a competition for the enzyme between ornithine and citrulline, analogous to the cases described by Frantz & Stephenson (1947), Huang & Niemann (1951) and Thomas, MacAllister & Niemann (1951) where one of the end-products of peptide hydrolysis was a competitive inhibitor. The data given in Table 8 confirm that the inhibition by ornithine is competitive. At a given ornithine concentration the inhibition increased the lower the citrulline concentration. A plot of the reciprocal of the substrate concentration (abscissa) against the reciprocal of the velocity (ordinate), according to Lineweaver & Burk (1934) gave the same intersect on the ordinate with and without ornithine, but the two curves had significantly different slopes.

Effect of ornithine decarboxylase

The inhibition of the arsenolysis by ornithine suggested that ornithine might also be responsible for the very low rate of phosphorolysis of citrulline. To test this, suspensions of *Esch. coli* containing an L-ornithine decarboxylase were added to the system (see Knivett, 1954*c*). Preliminary experiments showed that fresh liver suspensions and aqueous extracts of acetone-dried liver rapidly yield CO_2 on addition of *Esch. coli* in the absence of citrulline, due to the decarboxylation of amino acids in the liver extract and to the fermentation of carbohydrate. Dialysis of the liver extract was found to reduce these side reactions to a low value. As seen in Table 9, suspensions of *Esch. coli* greatly increase the rate of CO_2 evolution from citrulline. Allowing for the blank, the increase was about 20-fold and approached the rates observed in the presence of arsenate. When comparing the rates shown in Table 9 it must be borne in mind that ornithine decarboxylase doubled the total CO_2 production from citrulline.

Ornithine decarboxylase is known to be inhibited by hydroxylamine (Taylor & Gale, 1945). The last three horizontal lines of Table 9 show that although 0·01 M hydroxylamine inhibits the ornithine decarboxylase activity almost completely, the acceleration of the hydrolysis of citrulline by *Esch. coli* is not completely inhibited by hydroxylamine. This suggests that apart from ornithine decarboxylase *Esch. coli* suspensions contain a second substance, henceforth referred to as 'factor' which promotes the decomposition of citrulline. As heated suspensions of *Esch. coli* have no effect, the 'factor' is probably an enzyme.

Phosphate is an essential component in this system as illustrated by Table 10, whilst ATP and ADP have no effect. In control experiments it was noted that the decarboxylation of L-ornithine and L-lysine by *Esch. coli* was also increased by

Table 8. *Competitive inhibition of the arsenolysis of citrulline by ornithine*

Dialysed guinea pig liver suspension (stored for 6 weeks at −15°), 1 in 20 final concentration; 0·125 M arsenate buffer, pH 6·7; 40°; N_2; total vol., 4 ml. The data present the initial reaction rates.

Concentration of DL-citrulline (M)	Total CO_2 formed in 10 min. (μl.)	
	No ornithine	0·0125 M L-ornithine
0·1	121	61
0·05	109	39
0·025	78	21·8
0·0125	43·5	10·5
0·0063	23·7	5·3
0·0031	11·8	2·6

Table 9. *The effect of washed cells of* Esch. coli *on citrulline breakdown in the presence of guinea pig liver and phosphate*

Dialysed guinea pig liver homogenate, 1 in 20 final dilution; 0·125 M phosphate, pH 6·5, present throughout, unless otherwise stated; L-citrulline, 0·05 M; L-ornithine, 0·025 M; hydroxylamine, 0·01 M; *Esch. coli* ≡ 15 mg. dry wt; total vol., 2 ml.; 40°; N_2; 60 min.

Additions	CO_2 evolution (μl.)
No liver, *Esch. coli*, citrulline	9
Liver, *Esch. coli*	28·5
Liver, citrulline	30
Liver, *Esch. coli*, citrulline	430·5
Liver, *Esch. coli* (heated 10 min. at 100°), citrulline	28
Liver, no phosphate, citrulline, arsenate 0·125 M	312
Liver, *Esch. coli*, ornithine	754
Liver, *Esch. coli*, ornithine, hydroxylamine	11·5
Liver, *Esch. coli*, citrulline, hydroxylamine	122·5

Table 10. *Effect of phosphate on* CO_2 *evolution from citrulline in the presence of liver and* Esch. coli

Dialysed aqueous extract of acetone-dried sheep liver. 0·05 M L-citrulline; pH 6·8; *Esch. coli* ≡ 15 mg. dry wt.; total vol., 2 ml.; 40°; N_2.

Phosphate concentration (M)	CO_2 formed in 60 min. (μl.)
0	28
0·005	67
0·04	172·5
0·04 (no *Esch. coli*)	3
0·04 (no liver)	2

phosphate (Table 11), but this effect is relatively small compared with the effect on the decomposition of citrulline.

Effects of amino acid derivatives

The phosphorolysis of citrulline in the presence of *Esch. coli* and liver enzymes is accelerated by the addition of carbamyl and acetyl derivatives of amino acids (0·04 M). These substances were tested because Cohen & Grisolia (1950) and Grisolia & Cohen (1951, 1952, 1953) have shown that this type of compound can accelerate the aerobic conversion of ornithine into citrulline in mammalian liver. The acceleration of phosphorolysis is shown in Table 12. Effects were obtained with carbamylglutamate, acetylglutamate, carbamylglutamine, and carbamylalanine. They increased with time and were much larger during the second hour than during the first. No effects were observed with carbamylphenylalanine, carbamylleucine, acetylleucine, acetylalanine, carbamylaspartate, carbamylasparagine, or with glutamate, glutamine, aspartate and asparagine. It should be emphasized that no effects of the amino acid derivatives were found when either liver extract, or *Esch. coli* was omitted (see also Slade, 1953). Arsenolysis was not affected by the substances.

The effects recorded in Table 12 were obtained in the presence of hydroxylamine, i.e. in the absence of ornithine decarboxylase activity. They are therefore related to the action of the *Esch. coli* factor. Effects also occur in the absence of hydroxylamine (Table 13), but their relative magnitude is smaller, as they are superimposed on the large effect of ornithine decarboxylase.

Search for phosphorylated intermediates

Phosphorolysis or arsenolysis involves in some cases (Warburg & Christian, 1939; Bücher & Garbade, 1952) the intermediate formation of a phosphorylated or arsenolated compound. In an attempt to detect a phosphorylated intermediate, experiments of the type described in Tables 9 and 10 were repeated with radioactive phosphate (10 μc ^{32}P/flask). After various periods of incubation the medium was analysed chromatographically for phosphorylated compounds but no radioactive spot, apart from that for inorganic phosphate, appeared. When ATP and ADP were added no incorporation of radioactive phosphate into ATP or ADP was found. The latter experiments were carried out on washed liver mitochondria where the rate of decomposition of adenosine phosphates was slow.

Distribution of the enzyme systems responsible for the arsenolysis of citrulline

Brain, kidney, testis, lung, spleen, cardiac and striated muscle of the rat, pancreas and small intestine of the sheep, as well as pigeon liver were found to be completely inactive when tested under the conditions stated in Tables 1 and 2. The three mammalian livers tested (rat, guinea pig and sheep) as well as toad liver contained the enzyme system.

Table 11. *Effect of phosphate on the decarboxylation of* L-*ornithine and* L-*lysine by washed cells of* Esch. coli

Succinate buffer 0·1 M, pH 6·5; Na phosphate, pH 6·5; 0·025 M amino acid; *Esch. coli* ≡ 15 mg. dry wt.; total vol., 2 ml.; 40°; N_2.

Amino acid	Phosphate concentration (M)	CO_2 formed in 60 min. (μl.)
L-Ornithine	0	692
L-Ornithine	0·005	766
L-Ornithine	0·04	809
L-Lysine	0	726
L-Lysine	0·04	925

Table 12. *The acceleration by carbamyl- and acetyl-amino acids of citrulline breakdown in the presence of liver, phosphate,* Esch. coli *and hydroxylamine*

Aqueous extract of lysed sheep-liver mitochondria in Expts. 1–3; dialysed guinea pig liver suspension in Expt. 4; 0·1 M phosphate, pH 6·8; *Esch. coli* ≡ 15 mg. dry wt.; 0·01 M hydroxylamine; 0·04 M substrates; total vol., 2·5 ml.; 40°; N_2.

Expt. no.	Substrates	CO_2 liberated (μl.) 0–60 min.	60–120 min.	120–150 min.
1	L-Citrulline	81·5	47·5	18
	Carbamyl-L-glutamate	19·5	8	2
	Carbamyl-L-glutamate; L-citrulline	127	82	32
2	L-Citrulline	71	45·5	22·5
	Carbamyl-L-glutamine	8	3	6
	Carbamyl-L-glutamine; L-citrulline	124	87	41
3	L-Citrulline	44	24·5	11
	Acetyl-L-glutamate	3	4	2
	Acetyl-L-glutamate; L-citrulline	92	63	28
4	L-Citrulline	91	59	28
	Carbamyl-L-alanine	16	0	0
	Carbamyl-L-alanine; L-citrulline	133	89	38

Table 13. *Effect of acetyl-L-glutamate on the phosphorolysis of citrulline in the presence of liver extract, phosphate and* Esch. coli, *and absence of hydroxylamine*

Dialysed guinea pig liver homogenate; 0·143 M phosphate, pH 6·8; 0·029 M L-citrulline; *Esch. coli* ≡ 30 mg. dry wt.; total vol., 3·5 ml.; 40°; N_2.

Acetyl-L-glutamate concentration (M)	CO_2 liberated (μl.) after 60 min.	180 min.
0	188	439
0·0145	209·5	490
0·029	215·5	510
0·058	227·5	555

Arsenolysis was also found in micro-organisms for which 'arginine dihydrolase' activity has already been reported, e.g. *Clostridium welchii*, *Proteus vulgaris* and *Pseudomonas fluorescens*. Dried baker's yeast and brewer's yeast showed distinct, but weak arsenolysis (about 1 μl. CO_2/mg. dry weight/hr.).

The presence of the factor which accelerates the phosphorolysis of citrulline was tested in a system containing liver extract, phosphate buffer, citrulline and hydroxylamine (at concentrations and conditions as stated in Table 9). No activity was found in washed suspensions of *Lactobacillus arabinosus*, *Streptococcus faecalis*, *Bacillus proteus vulgaris*, *Clostridium welchii* SR 12, *Bacillus subtilis*, *Sarcina lutea* and *Bacterium faecalis alkaligenes*, when these organisms were grown on the same medium in which *Esch. coli* produced the factor. When the amino acid hydrolysate was omitted from the medium *Esch. coli* failed to produce the factor.

DISCUSSION

Comparison of citrulline decomposition in bacteria and in liver

The experiments reported in this paper demonstrate the occurrence in mammalian liver of an enzyme system which can decompose citrulline to ornithine, carbon dioxide and ammonia by arsenolysis or phosphorolysis. The enzyme system is very similar to that previously found in micro-organisms. Of the three reactions (1), (2) and (3), which take place in bacteria (to be regarded as different manifestations of the same basic enzyme system), so far only (2) and (3) have been found in liver.

The fact that the distribution of the enzyme system in animal tissues parallels the occurrence of the urea-synthesizing enzymes suggests that the two are associated, the degrading enzymes being components of the systems which under physiological conditions are responsible for the synthesis. The conditions required for arsenolysis and phosphorolysis are usually not realized in living cells, as arsenate is absent and the physiological concentrations of the reactants are too low for phosphorolysis. Another factor favouring synthesis of citrulline from ornithine under physiological conditions is the inhibition of phosphorolysis by ornithine.

Arginine is known in micro-organisms to be synthesized from ornithine via citrulline, e.g. in *Neurospora crassa* (Srb & Horowitz, 1944), in *Ophiostoma multiannulatum* (Bergström & Sjöbeck, 1950) in *Esch. coli* (Davis, 1953) and *Lb. arabinosus* (Hood & Lyman, 1950; Ory, Hood & Lyman, 1954). The bacterial enzymes decomposing citrulline may be looked upon as being connected with the synthesis of arginine in the same manner as the corresponding hepatic enzyme system is connected with the formation of urea. In those micro-organisms which possess the arginine dihydrolase component, reactions of this system have evolved to serve as an energy-giving process (reaction 1) if the substrate is available in the medium.

Mechanism of arsenolysis and phosphorolysis

Warburg & Christian (1939), who studied arsenolysis in the triosephosphate dehydrogenase system, assumed the formation of an arsenolated intermediate, arsenophosphoglycerate, replacing the normal phosphorylated intermediate, diphosphoglycerate (see also Racker & Krimsky, 1952). The formation of arsenolated intermediates (glucose 1-arsenate and acetylarsenate) was also assumed by Doudoroff, Barker & Hassid (1947) and Stadtman & Barker (1950) to explain the arsenolysis of glucose 1-phosphate and acetylphosphate. Recently the view has gained ground that effects of phosphate in some cases may be due to the phosphorylation of an enzyme rather than of an intermediate (Webster & Varner, 1954; Lipmann, 1954; Boyer & Harrison, 1954; Snoke, 1953). In such cases arsenolysis is expected to be due to combination of arsenate with the enzyme.

The facts which are to be taken into consideration by any hypothesis attempting to account for the arsenolysis and phosphorolysis of citrulline are as follows.

(*a*) Arsenolysis requires only substrate, enzyme and arsenate, in both liver and bacteria.

(*b*) Phosphorolysis, in order to proceed at an appreciable rate, requires not only phosphate, substrate and enzyme but also removal of ornithine by ornithine decarboxylase (in liver and bacteria) or removal of phosphate by transfer to ADP (in bacteria).

(*c*) Ornithine inhibits competitively both arsenolysis and phosphorolysis.

(*d*) Arsenolysis is competitively inhibited by phosphate.

(*e*) High concentrations of citrulline, phosphate and arsenate are required for appreciable reaction rates.

The following inferences must also be taken into consideration in formulating a hypothesis.

(*f*) The competition between ornithine and citrulline indicates that the two substances react with the same group of the enzyme.

(*g*) Competition between phosphate and arsenate indicates that these two substances also react with a common group of the enzyme. The points of attachment to the enzyme of the pairs phosphate–arsenate and citrulline–ornithine are not the same.

The following reaction mechanism, which is in some respects analogous to that proposed by Webster & Varner (1954) for the synthesis of glutamine and of Snoke (1953) for the synthesis of glutathione, is in accordance with these facts and inferences. It is assumed that the enzyme (*E*) can combine reversibly with citrulline, and possibly also with ornithine:

$$E + \text{citrulline} \rightleftharpoons E\text{–citrulline}, \qquad (4a)$$

$$E + \text{ornithine} \rightleftharpoons E\text{–ornithine}. \qquad (4b)$$

The enzyme–citrulline complex, by reaction with phosphate or arsenate is assumed to be split as follows:

$$E\text{–citrulline} + \text{phosphate} \rightleftharpoons E\begin{matrix}\diagup\text{phosphate}\\ \diagdown\text{citrulline}\end{matrix} \rightarrow$$

$$E\begin{matrix}\diagup\text{phosphate}\\ \diagdown\text{ornithine}\end{matrix} + CO_2 + NH_3 \rightleftharpoons E\text{–phosphate} + \text{ornithine}. \qquad (5a)$$

$$E\text{–citrulline} + \text{arsenate} \rightleftharpoons E\begin{matrix}\diagup\text{arsenate}\\ \diagdown\text{citrulline}\end{matrix} \rightarrow$$

$$E\begin{matrix}\diagup\text{arsenate}\\ \diagdown\text{ornithine}\end{matrix} + CO_2 + NH_3 \rightleftharpoons E\text{–arsenate} + \text{ornithine}. \qquad (5b)$$

The centre stages of (*5a*) and (*5b*), it should be noted, are written as being irreversible, although $E\begin{matrix}\diagup\text{phosphate}\\ \diagdown\text{citrulline}\end{matrix}$ and $E\begin{matrix}\diagup\text{phosphate}\\ \diagdown\text{ornithine}\end{matrix}$ may be interconvertible by the exchange reaction

$$E\begin{matrix}\diagup\text{phosphate}\\ \diagdown\text{citrulline}\end{matrix} + \text{ornithine} \rightleftharpoons E\begin{matrix}\diagup\text{phosphate}\\ \diagdown\text{ornithine}\end{matrix} + \text{citrulline}. \qquad (6)$$

The competitive inhibition by ornithine is either due to (*4b*) or to (6). The reactions (*5a*), though not reversible as a whole, can be brought to a standstill either by the competitive inhibition of ornithine, or by blocking the enzyme in the form of *E*–phosphate. Hence either the removal of ornithine by ornithine decarboxylase or the transfer of phosphate from *E*–phosphate to ADP

$$E\text{–phosphate} + \text{ADP} \rightleftharpoons E + \text{ATP} \qquad (7)$$

promotes (*5a*) from left to right.

To explain the phosphorolytic effect of high concentrations of phosphate (Table 1) and of ornithine decarboxylase (Table 9) it must be assumed that *E*–phosphate is capable of undergoing hydrolysis at a rate increasing with its concentration. Removal of ornithine by ornithine decarboxylase, or high concentrations of inorganic phosphate both cause the postulated increase in the concentration of *E*–phosphate.

To shift reaction (*5b*) from left to right a rapid decomposition of *E*–arsenate is postulated, in accordance with the general experience that arsenate esters of physiological substances are less stable than the corresponding phosphate esters.

The virtual non-occurrence of reactions (*4a*) and (*5a*) under physiological conditions is accounted for by the fact that the relative affinity of the enzyme for citrulline is low, and by the inhibition by ornithine.

The formation of a phosphorylated substrate (Oginsky & Gehrig, 1953; Knivett, 1954*b*) according to the scheme

$$E\text{–citrulline} + \text{phosphate} \rightarrow E + \text{citrulline-phosphate},$$

is unlikely (though not ruled out) because of the failure to detect a phosphorylated substrate.

As the phosphorolysis of citrulline can be coupled (in bacteria) with the synthesis of ATP from ADP and phosphate, an energy-rich phosphate group must arise at some intermediate stage. The energy-rich phosphate is taken to be the phosphorylated enzyme (*E*–phosphate) formed at stage (*5a*).

Since the reactions discussed here do not occur under physiological conditions, those in which phosphate takes part are assumed to be stages of citrulline synthesis in the living cell. This synthesis is initiated by reaction (7) and followed by a modified form of (*5a*) and completed by (*4a*), all three reactions proceeding from right to left. The modification of (*5a*) occurs at the irreversible centre step. This is replaced by other reactions in which the carbamyl group is built up and transferred to the enzyme-bound ornithine. The carbamyl donor is presumably compound X of Grisolia (1951). The arsenolysis or phosphorolysis do not require an acceptor for the carbamyl group which is taken as indicating that the mechanism of the fission of the carbamyl group of citrulline differs from that of the synthesis.

SUMMARY

1. Citrulline, whilst stable in suspensions of fresh liver or extracts of acetone-dried liver, is readily split into ornithine, CO_2 and ammonia if arsenate is added. This reaction is analogous to the arsenolysis of citrulline in *Streptococcus faecalis* (Knivett, 1954*b*).

2. A slow fission of citrulline occurs in the presence of high concentrations (0·05 M and above) of orthophosphate.

3. Some properties of the enzyme system responsible for the arsenolysis have been studied. The pH optimum of the arsenolysis is at 6·7. The Michaelis constant varies with experimental conditions between 0·01 and 0·05.

4. Phosphate inhibits competitively the action of arsenate.

5. The arsenolytic ability of liver preparations is not reduced by prolonged dialysis. Ornithine specifically inhibits arsenolysis and phosphorolysis. The inhibition is competitive.

6. Phosphorolysis is greatly accelerated by the addition of washed suspensions of *Esch. coli*, which contain ornithine decarboxylase. Hydroxylamine, which inhibits ornithine decarboxylase does not fully abolish the accelerating effect of *Esch. coli*. It is concluded that a factor in addition to ornithine decarboxylase promotes phosphorolysis. Its nature is unknown.

7. Carbamyl and acetyl derivatives of amino acids do not affect the rate of arsenolysis but some compounds of this group (carbamylglutamate, carbamylglutamine, carbamylalanine and acetylglutamate) accelerate the rate of phosphorolysis in the presence of ornithine decarboxylase or the factor of *Esch. coli*.

8. Among animal tissues liver was the only one in which arsenolysis of citrulline was found. Avian liver was inactive. It is assumed that the enzymes responsible for arsenolysis are components of the system which synthesizes citrulline in the living cell.

9. A mechanism of arsenolysis and phosphorolysis is proposed. It involves the formation of a phosphorylated or arsenolated enzyme.

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The Formation of Phosphopyruvate in Washed Particles of Kidney and Liver

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A formation of phosphopyruvate from C_4 dicarboxylic acids was first observed by Kalckar (1939). His findings were confirmed by Leloir & Munoz (1944) and Bartley (1954); adenosine triphosphate (ATP) was shown to be the phosphate donor (Bartley, 1954). In the present paper the conditions for the formation of phosphopyruvate have been studied in detail.

EXPERIMENTAL

Tissue preparations. These were usually twice-washed homogenates, i.e. mixtures of mitochondria and nuclei ('cyclophorase') prepared according to Bartley & Davies (1954). Sheep kidney was dispersed with a Waring Blendor; rat and pigeon liver were homogenized in a stainless-steel homogenizer of the Potter–Elvehjem type. In a few experiments washed mitochondria were used; these formed about the same amount of phosphopyruvate as the cyclophorase preparations: the latter were used mainly because of the greater ease of preparation.

Media. In most experiments 2 ml. of the suspension (about 70 mg. dry wt.) were added to 2 ml. of a solution containing K phosphate buffer pH 7·4 (0·02 M), Na adenosine triphosphate (0·002 M), $MgCl_2$ (0·002 M) and the oxidizable substrate, the amounts of which are given in the description of the individual experiments.

Special chemicals. ATP was prepared according to LePage (1949), phosphopyruvate according to Ohlmeyer (1951), 2-phosphoglyceric acid according to Kiessling (1935), phosphomalate according to Friedkin & Lehninger (1947), 3-phosphoglyceric acid according to Neuberg & Lustig (1942). Hydroxymaleic and hydroxyfumaric acids were made by the following modification of the method of Fenton & Jones (1900). A solution of DL-malic acid (26·8 g.) and ferrous sulphate (7 g.) in water (60 ml.) was frozen by standing the container in a mixture of solid CO_2 and ethanol. The mixture was vigorously stirred during freezing in order to prevent the formation of one solid block. Hydrogen peroxide (100 ml. of 20 vol.) was frozen in a similar manner and added in small portions to the mush of malic acid and $FeSO_4$ solution. If hydroxymaleic was required, the procedure was then as described by Fenton & Jones, i.e. ether extraction from 10% (w/v) H_2SO_4 solution of the crude product. To obtain hydroxyfumaric acid the mixture was added to ice-cold H_2SO_4 (final concentration of H_2SO_4, 30% w/v) in small portions. The mixture was allowed to warm to room temperature and then heated for 30 min. on a water bath at 40°. After cooling to room temperature the mixture was shaken with successive 50 ml. portions of ether (five or six extractions). The combined ether extracts were evaporated to dryness and the yellowish solid obtained was taken up in the minimum amount of warm acetone and reprecipitated by the addition of 3 times its volume of $CHCl_3$. The crude product (m.p. 168°) was dissolved in the minimum amount of boiling ethyl propionate and treated with charcoal. After filtration, while still hot, the substance was induced to crystallize by the gradual addition of $CHCl_3$ (about 3 vol.). Recrystallization from ethyl propionate and $CHCl_3$ gave hydroxyfumaric acid (m.p. 176° uncorr.). Both hydroxymaleic acid (m.p. 152°) and hydroxyfumaric acids were estimated by the aniline decarboxylation method (Krebs & Eggleston, 1945), according to which the hydroxyfumaric acid was 98·5% and the hydroxymaleic acid 96% pure. Another sample of hydroxymaleic acid was prepared and recrystallized (m.p. 151–152°) according to the method of Roberts (1952). Half of this material was dissolved in 30% (w/v) H_2SO_4 warmed to 40° for 30 min., cooled and extracted into ether. After evaporation of the ether the solid was dissolved in the minimum of acetone at 60° and treated with charcoal. The hydroxyfumaric acid was precipitated by the addition of 3 vol. of light petroleum (b.p. 60–80°) filtered off, dissolved in cold acetone and precipitated by light petroleum several times until the m.p. was 178°. The hydroxyfumaric acid was then twice recrystallized from ethyl acetate by the addition of light petroleum and dried.

Oxaloacetate in this paper refers to the equilibrium mixture of keto and enol forms produced when hydroxymaleic acid was dissolved in water, neutralized to pH 7·4, and allowed to stand for at least 5 min.

Other oxidizable substrates were commercial preparations; malic acid was added as the racemic mixture. Unless otherwise stated, substrates were neutralized to pH 7·4 with N-NaOH before addition to the incubation medium. Other chemicals were of 'AnalaR' standard.

Analytical methods. The phosphorus-containing compounds were separated by paper chromatography, and determined as described by Bartley (1953).

The sum of (malate + fumarate) was estimated with washed suspensions of *Lactobacillus arabinosus* (Nossal, 1952) and keto acids by the 'specific extraction' procedure of Friedemann & Haugen (1943). Oxygen uptake was measured manometrically in the Warburg apparatus. A stick of yellow phosphorus was placed in the centre well in anaerobic experiments.

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Table 1. *Comparison of phosphopyruvate formation from different substrates*

Expt. 1. Rat-liver cyclophorase, about 60 mg. dry wt., incubated in Warburg vessels at 40° in O_2 for 15 min. Expt. 2. Pigeon-liver cyclophorase, about 60 mg. dry wt., incubated at 20° for 20 min. Each vessel contained 2 ml. of the tissue suspension in 0·9% (w/v) KCl and 2 ml. of a solution containing K phosphate buffer pH 7·4 (0·02 M), Na adenosine triphosphate (0·002 M), $MgCl_2$ (0·002 M), 0·025 M substrate and in Expt. 1 tracer amounts of $^{32}PO_4$. The centre well contained 0·2 ml. of 2 N-NaOH and filter paper. The phosphopyruvate formed in Expt. 1 was measured by determination of its radioactivity; in Expts. 2 and 3 the phosphopyruvate was measured, after isolating the substance on the paper chromatogram, by the phosphate content of the chromatogram spots.

Expt. no.	Tissue	Substrate	Oxygen consumption (μmoles)	Phosphopyruvate formed (μmoles)
1	Rat liver	Citrate	37·3	1·4
		α-Oxoglutarate	38·7	1·0
		Succinate	30·5	2·1
		Fumarate	18·9	3·2
		Malate	21·0	2·7
2	Pigeon liver	Malate	—	6·3
		Oxaloacetate	—	4·1
3	Pigeon liver	Malate	5·0	5·5
		Oxaloacetate	6·0	3·6

Measurements of radioactivity were made in a liquid counter (M6, 20th Century Electronics, Dunbar Street, West Norwood, London, S.E. 27) with the diluted paper digests. Counts were corrected for decay, background and dead time.

Calculation of amounts of phosphate esters from measurements of radioactivity. In the tissue suspensions used the ATP exchanged its labile phosphate groups with inorganic P very rapidly and within a few minutes specific activities became equal to that of the inorganic phosphate. Any organic P formed from ATP or directly from inorganic P thus had the same specific activity as P and the quantities of the organic P formed could be calculated by measuring the radioactivity of the substances after separation.

RESULTS

Comparison of phosphopyruvate formation from citrate, α-oxoglutarate, succinate, fumarate or malate. Earlier experiments had shown qualitatively (Bartley, 1954) that phosphopyruvate is formed when any tricarboxylic acid cycle intermediate or pyruvate and bicarbonate was incubated with respiring sheep-kidney cyclophorase preparations. The experiments recorded in Table 1 were designed to measure quantitatively the yield of phosphopyruvate in the presence of various precursors. Expt. 1 shows that during 15 min. of incubation the greatest quantity of phosphopyruvate was formed when fumarate or malate was the substrate; oxaloacetate was not tested in this experiment, but as shown by Expts. 2 and 3, and Table 4, it was rather less active in forming phosphopyruvate than malate or fumarate. This is discussed more fully later. Pigeon liver (about 60 mg. dry wt.) incubated at 20° for 20 min. accumulated about twice as much phosphopyruvate from malate (about 6 μmoles) as the same amount of rat liver incubated for 15 min. at 40° (about 3 μmoles). The amounts of phosphopyruvate (4–5 μmoles) formed from fumarate were not markedly altered by variation of the substrate concentration between 0·01 and 0·04 M (Table 2). As described by Bartley (1954), other phosphate esters accumulate when incubation is prolonged. The main additional chromatogram spot (a mixture of 2 or 3 phosphoglyceric acids, see later) appears when the amount of phosphopyruvate becomes large. In the experiment described in Table 2 the final amounts of phosphoglyceric acids were 3–3·5 μmoles. The molar sum of phosphopyruvate and phosphoglycerates was about equivalent to half the molar amount of O_2 utilized (Table 2).

Table 2. *Comparison of phosphopyruvate and phosphoglycerate formation with varying concentrations of fumarate*

Rat-liver cyclophorase, about 50 mg. dry wt., incubated in Warburg vessels for 80 min. in O_2 at 20°. Each vessel contained 2 ml. of the tissue suspension in 0·9% (w/v) KCl and 2 ml. of a solution containing K phosphate buffer pH 7·4 (0·02 M), Na adenosine triphosphate (0·002 M), $MgCl_2$ (0·002 M) and substrate. The centre well contained 0·2 ml. of 2 N-NaOH and filter paper. The phosphate-containing compounds were measured, after isolation on the paper chromatogram, by determination of the phosphate content of the chromatogram spots.

Fumarate concentration (M)	O_2 uptake (μmoles)	Phosphopyruvate formed (μmoles)	2- and 3-Phosphoglycerates formed (μmoles)
0·04	11·40	4·9	3·0
0·02	12·75	4·8	3·0
0·01	13·90	4·2	3·5

Anaerobic formation of phosphopyruvate from fumarate, malate or oxaloacetate. Under anaerobic conditions, with fumarate or malate as substrates, and fluoride (0·02 M) as an inhibitor of adenosine-

triphosphatases, the maximum phosphopyruvate formation was less than 10% of the aerobic formation, e.g. 70 mg. dry wt. of tissue particles formed 0·3 μmole in 1 hr. Oxaloacetate yielded much more phosphopyruvate under these conditions (Table 3). The maximum amount of phosphopyruvate was found after 10 min. of incubation; if incubation was prolonged the substance disappeared. This was probably correlated with the decline of ATP concentration, which occurred in spite of the fluoride which was added to the incubation medium and the action of pyruvic phosphokinase. No phosphoglycerates were formed, presumably because of the inhibition of enolase by the fluoride.

Comparison of the aerobic formation of phosphopyruvate from malate or oxaloacetate. The relatively low rate of phosphopyruvate formation from oxaloacetate might be due to an inhibition by a high concentration of oxaloacetate. To test this, three flasks containing pigeon-liver cyclophorase and cofactors were set up; to one were added 25 μmoles of malate, to the second 25 μmoles of oxaloacetate and to the third oxaloacetate was added in portions of 0·5 μmole at regular intervals over a period of 1 hr. to a total of 25 μmoles. The results given in Table 4 show that the rate of phosphopyruvate formation was higher at the higher oxaloacetate concentration. Thus oxaloacetate in the concentra-

Table 3. *Anaerobic formation of phosphopyruvate from oxaloacetate*

Sheep-kidney cyclophorase, about 60 mg. dry wt., incubated in Warburg vessels at 40° in N_2 with yellow phosphorus in the centre well. The 4 ml. of incubation mixture contained 2 ml. of the tissue suspension in 0·9% (w/v) KCl and 2 ml. of a solution containing K phosphate buffer pH 7·4 (about 20 μmoles), Na adenosine triphosphate (about 5 μmoles), $MgCl_2$ (0·002 M), NaF (0·04 M) and oxaloacetate 0·025 M. The phosphate-containing compounds were measured after isolation on the paper chromatogram by determination of the phosphate content of the chromatogram spots.

Incubation time (min.)	ATP + ADP phosphate present (μmoles)	AMP phosphate present (μmoles)	Orthophosphate present (μmoles)	Phosphopyruvate formed (μmoles)
0	17·2	0	23·9	0
5	8·5	2·7	26·9	1·0
10	6·7	3·3	29·5	1·5
20	5·2	4·3	30·6	1·5
40	—	—	—	0

Table 4. *Comparison of phosphopyruvate formation from malate or oxaloacetate*

Pigeon-liver cyclophorase shaken in open flasks at 20°. Each flask contained 10 ml. of pigeon-liver cyclophorase in 0·9% (w/v) KCl, and in Expts. 1 and 2, 10 ml. of a medium containing K phosphate buffer pH 7·4 (0·02 M), Na adenosine triphosphate (0·002 M), $MgCl_2$ (0·002 M) and 0·025 M substrate. In Expt. 3 the 10 ml. of cyclophorase was added to 9·02 ml. of a medium which contained initially only 2% of the amount of substrate but which contained the same total quantity of the other cofactors. The remainder of the substrate was added in this experiment in portions of 0·02 ml. at 1 min. intervals throughout the incubation until the 500 μmoles of substrate had been added. In Expt. 1 the substrate was malate and in Expts. 2 and 3 it was oxaloacetate. 2·0 ml. samples of the incubation mixture were removed at the times stated below for analysis. The analytical data refer to the content in the 2 ml. sample.

Expt. no.	Incubation time (min.)	Malate present (μmoles)	Oxaloacetate present (μmoles)	Phospho-pyruvate found (μmoles)	2- and 3-Phospho-glycerates found (μmoles)	Ratio: total μmoles substrate phosphorylated/ substrate not recovered as malate or keto acid × 100
1	0	25	0	0	0	—
	15	18·8	0·52	2·15	1·1	57·2
	30	15·9	0·95	2·8	2·0	59·4
	45	14·0	0·64	—	—	—
	60	12·9	0·87	1·8	5·0	60·5
2	0	0	25	0	—	—
	15	3·52	18·0	1·2	1·1	63·3
	30	2·29	11·2	1·75	1·8	30·8
	45	1·32	7·1	—	—	—
	60	1·24	4·7	0·8	2·95	19·6
3	0	0	0·5	—	—	—
	15	0·22	4·47	0·25	0·3	—
	30	0·66	6·21	0·45	0·90	—
	45	2·49	10·3	—	—	—
	60	0·98	11·9	0·5	1·6	—

tion tested, did not inhibit phosphopyruvate formation. A remarkably high proportion (over 50%) of the malate removed can be accounted for as phosphorylated products. In agreement with other experiments the formation of phosphopyruvate from oxaloacetate was lower than that with malate.

Phosphopyruvate formation in the presence of ketone-binding agents. Ketone-binding agents were added in order to test whether free oxaloacetate was an intermediate in the phosphopyruvate formation from malate. Table 5 shows the effect of semicarbazide on phosphopyruvate formation from malate by pigeon liver. In the first experiment phosphorylation was slightly reduced by the semicarbazide, but as the respiration was also inhibited in roughly the same proportion, this might be expected. In the second experiment the phosphorylation was slightly higher in the presence of the semicarbazide.

The experiments were repeated in the presence of other ketone-binding agents (hydroxylamine 0·005M or cyanide 0·01M); ferricyanide (0·01M) was added with the cyanide as hydrogen acceptor. Table 6 correlates oxygen and malate consumption and production of phosphopyruvate and of phosphoglycerate. Both semicarbazide and hydroxylamine approximately halved the oxygen uptake without greatly reducing either the malate consumption or the formation of phosphopyruvate and phosphoglycerate. With cyanide the malate consumption was approximately halved. Of special interest is the almost quantitative recovery of malate as phosphorylated products in the presence of potassium cyanide. Raising the cyanide concentration to 0·02M reduced the phosphorylation to about one-third. When oxaloacetate was the substrate no phosphopyruvate was formed in the presence of semicarbazide or of cyanide.

Phosphomalate as intermediate in the formation of phosphopyruvate. Lipmann (1941) suggested that phosphomalate is an intermediate in the formation of phosphopyruvate from C_4 acids. Phosphomalate was prepared by Friedkin & Lehninger (1947) and according to Friedkin (1951) phosphomalate is not oxidized by liver and does not inhibit the oxidation of malate. It was confirmed that no oxygen uptake occurs when phosphomalate is the sole substrate, but 0·0025M phosphomalate was found to reduce oxygen uptake and phosphorylation to about one-third when malate was the substrate (Table 7). To test the possibility of phosphomalate acting as an intermediate in phosphopyruvate formation, 2 μmoles of the substance were added to 4 ml. of a pigeon cyclophorase preparation oxidizing malate; isotopic phosphate was added to the incubation mixture which was shaken at 20° for 1 hr. The phosphorylated substances were separated on the paper chromatogram and their radioactivities were determined. The phosphopyruvate and phosphoglycerate formed were both in isotopic equilibrium with the inorganic phosphate, whereas the phosphomalate was inactive. Thus phosphomalate cannot be an intermediate in the formation of phosphopyruvate.

Table 5. *Phosphorylated products formed from malate with and without the addition of semicarbazide*

Pigeon-liver cyclophorase incubated in Warburg vessels in O_2 at 20°. Expt. 1: 0·0125M substrate, 84 mg. dry wt. of particles. Expt. 2: 0·0062M substrate, 0·02M THAM buffer pH 7·4, 96 mg. dry wt. of particles. Other additions as in Table 1.

Expt. no.	Addition	O_2 uptake (μmoles)	Phospho-pyruvate formed (μmoles)	2- and 3-Phospho-glycerates formed (μmoles)
1	None	23·8	7·62	6·9
	Semicarbazide (0·04M)	18·5	3·98	6·05
2	None	—	5·46	1·63
	Semicarbazide (0·08M)	—	6·20	1·41

Table 6. *Phosphopyruvate formation from malate in the presence of ketone-binding agents*

Pigeon-liver cyclophorase incubated in Warburg vessels in O_2 at 20° for 1 hr. Malate initially present, 41·3 μmoles. The vessel with cyanide contained 0·01M ferricyanide as hydrogen acceptor; other additions as in Table 1.

Ketone-trapping agent added	O_2 consumption (μmoles)	Phospho-pyruvate formed (μmoles)	2- and 3-Phospho-glycerates formed (μmoles)	Malate consumption (μmoles)	% of malate consumption recovered as phosphorylated compounds
None	21·8	7·78	5·40	17·5	75·5
Semicarbazide (0·08M)	11·1	8·85	3·42	17·7	69·5
Hydroxylamine (0·005M)	12·95	4·38	4·65	14·7	63·4
Cyanide (0·01M)	0	3·72	5·08	9·1	96·7

Comparison of reactivity of enol and keto oxaloacetate. The dehydrogenation of malate might produce one of the enol forms of oxaloacetate, hydroxyfumaric or hydroxymaleic acid, rather than the keto form. These three substances are mutually interconvertible:

hydroxymaleic acid ⇌ oxaloacetic acid
⇌ hydroxyfumaric acid,

but the rate of interconversion is unknown.

Some samples of hydroxymaleic and hydroxyfumaric acids showed differences in the rates at which they formed phosphopyruvate; these differences were reduced, though not abolished, when the hydroxyfumaric acid was recrystallized. This suggested that the sample of hydroxyfumaric acid might contain an inhibitor. Accordingly, another sample of hydroxymaleic acid was prepared and recrystallized (m.p. 151–152°) by the method of Roberts (1952). Half of this was converted into hydroxyfumaric acid as described in the Experimental section and these purified samples of hydroxymaleic and hydroxyfumaric acids were used in the following experiment. To avoid inaccuracies due to the use of individual incubation vessels, samples were abstracted from a common incubation mixture. Four conical flasks of capacity 100 ml. were used. Each flask contained 49·5 mg. of the keto acid, one pair hydroxymaleic acid, one pair hydroxyfumaric acid. To one of each pair were added 15 ml. of a medium containing the usual cofactors and sufficient NaOH to neutralize the acid. The medium also contained aminotrishydroxymethylmethane (THAM) buffer (final conc. in medium 0·05M). The two flasks were shaken in a water bath at 20° for 6 min. to allow the keto–enol equilibrium to be established. Pigeon-liver cyclophorase suspension (15 ml.) was then added to each flask without stopping the shaking and a 4 ml. sample was immediately abstracted and pipetted into 0·5 ml. 30% (w/v) trichloroacetic acid TCA. Further samples were withdrawn after 2, 4, 6, 8 and 10 min. incubation. The other two flasks were treated in the same way, except that the cyclophorase was added immediately after the medium, without the 6 min. equilibration period. The TCA extracts were analysed for malate and phosphopyruvate. The results in Table 8 show that both hydroxymaleic

Table 7. *Comparison of oxygen uptake, malate consumption and phosphopyruvate formation with and without phosphomalate*

Pigeon-liver cyclophorase incubated at 20° in O_2 for 1 hr. Malate added 41·3 μmoles. Phosphomalate in Expt. 1, 0·0025M; in Expt. 2, 0·0005M. Dry weight of particles added, 84 and 75 mg. dry wt. respectively; other additions as in Table 1.

Expt. no.	Addition	O_2 consumption (μmoles)	Malate consumption (μmoles)	Phosphopyruvate formed (μmoles)	2- and 3-Phosphoglycerates formed (μmoles)
1	None	24·8	—	7·6	6·9
	Phosphomalate (0·0025M)	7·9	—	3·2	1·8
2	None	21·8	17·5	7·78	5·4
	Phosphomalate (0·0005M)	20·9	15·2	9·58	4·08

Table 8. *Comparison of phosphopyruvate and malate production from hydroxymaleic acid, hydroxyfurmaric acid and oxaloacetic acid formed from both these substances*

49·5 mg. of hydroxymaleic acid were weighed into each of a pair of 100 ml. conical flasks and the same amount of hydroxyfumaric acid into a further pair. To one of each pair of flasks were added 15 ml. of a medium containing K phosphate buffer pH 7·4 (0·02M), Na adenosine triphosphate (0·002M), $MgCl_2$ (0·002M), THAM buffer pH 7·4 (0·05M) and sufficient NaOH to neutralize the keto acid. The four flasks were shaken at 20° for 6 min. and medium was then added to the remaining two flasks. Pigeon-liver cyclophorase (15 ml.; 400 mg. dry wt.) was then immediately added to each flask. A 4 ml. sample of the mixture was immediately abstracted from each flask for analysis and further samples at the times stated below. The analytical values refer to content in the 4 ml. sample abstracted.

	Phosphopyruvate formed from				Malate formed from			
Time (min.)	Hydroxymaleic acid (μmoles)	Hydroxyfumaric acid (μmoles)	Oxaloacetic acid from hydroxymaleic acid (μmoles)	Oxaloacetic acid from hydroxyfumaric acid (μmoles)	Hydroxymaleic acid (μmoles)	Hydroxyfumaric acid (μmoles)	Oxaloacetic acid from hydroxymaleic acid (μmoles)	Oxaloacetic acid from hydroxyfumaric acid (μmoles)
2	0·74	0·58	1·00	0·92	0·44	0·24	0·64	0·46
4	1·20	1·32	1·68	1·76	1·26	1·00	1·36	1·26
6	1·60	1·52	2·10	2·20	1·72	1·28	1·82	1·88
8	2·08	1·86	2·66	2·86	2·16	2·04	2·50	2·46
10	2·32	2·50	2·92	2·90	2·68	2·82	3·08	2·96

and hydroxyfumaric acids formed phosphopyruvate at the same rate which was a little slower than the rate of phosphopyruvate formation from oxaloacetate. This would suggest that the keto form is the reactive tautomer. No clear distinction could be made in the rate at which malate was formed from the four substances.

Phosphorylated products formed from phosphopyruvate. The following evidence shows that the substance already referred to as 2- and 3-phosphoglycerates arises from phosphopyruvate.

A series of Warburg vessels were set up containing in the side arm about 3 μmoles of phosphopyruvate and 50 μmoles of fumarate and in the main compartment rat-liver cyclophorase, 1 μmole of fumarate, the cofactors previously stated and isotopic orthophosphate. After 10 min. incubation in oxygen at 20° the contents of the side arm were tipped in, one of the vessels was removed and the reaction stopped by the addition of TCA. By this time all the exchangeable phosphate had equilibrated. The other vessels were incubated for a further 2, 4, 6, 8, 10, 15, 20, 30 or 60 min. The radioactivity incorporated into the phosphopyruvate and 2- and 3-phosphoglycerates at the various times was measured and the total quantities of each substance were estimated. Table 9 shows that in this experiment the disappearance of phosphopyruvate after 60 min. incubation was exactly balanced by the formation of 2- and 3-phosphoglycerates. Moreover, the specific activity of the 2- and 3-phosphoglycerates was lower at the end of the incubation than at the time when inactive phosphopyruvate was added. This fall in specific activity could occur only if non-isotopic phosphate from phosphopyruvate was incorporated into 2- and 3-phosphoglycerates. Table 9 also shows that the formation of phosphopyruvate from fumarate was inhibited by the presence of preformed phosphopyruvate, presumably by mass action. New formation of phosphopyruvate did not occur until the initial concentration had fallen to about one-half. In a similar experiment summarized in Table 10 phosphopyruvate was added to a rat-liver suspension oxidizing citrate and measurements were made of the changes in the amount and specific activity of 2- and 3-phosphoglycerates with time. The amount of 2- and 3-phosphoglycerates increased with time, but the specific activity fell. As phosphopyruvate was the only source of non-isotopic phosphate, the decrease in specific activity of 2- and 3-phosphoglycerates means that it must have been formed from phosphopyruvate. Further evidence that 2- and 3-phosphoglycerates are formed from phosphopyruvate is presented in Table 11. It shows once again that when non-labelled phosphopyruvate is added to the incubation medium 2- and 3-phosphoglycerates contain phosphate of lower specific activity than the inorganic phosphate in the incubation medium. When 3-phosphoglyceric acid was added, the final product isolated in the 2- and 3-phosphoglycerates area of the chromatogram was radioactive, but the specific activity of the phosphopyruvate was less

Table 9. *Formation of 2- and 3-phosphoglycerates from phosphopyruvate by rat-liver cyclophorase*

Rat-liver cyclophorase incubated in Warburg vessels at 20° in O_2. The side arms of the vessels contained about 3 μmoles of phosphopyruvate and 50 μmoles of fumarate in 0·2 ml. water. The main compartments contained 2 ml. of cyclophorase (70 mg. dry wt.) in 0·9% (w/v) KCl, 1 μmole of fumarate, 40 μmoles of phosphate containing tracer amounts of $^{32}PO_4$, 4 μmoles of Na adenosine triphosphate and 4 μmoles of $MgCl_2$. Final volume 4 ml. The centre well contained 0·2 ml. of 2N-NaOH and filter paper. The vessels were incubated for 10 min. before the contents of the side arm were tipped; specific activities of orthophosphate, ATP and phosphopyruvate present after the 10 min. incubation but before tipping in the side arm contents are assumed to be the same, 3865 counts/min./μg. P. At the end of the incubation the specific activity of the phosphopyruvate was 1428 counts/min./μg. P and of 2- and 3-phosphoglycerates 931 counts/min./μg. P. The amount of radioactive material is calculated by dividing the total counts present in the isolated material at any time by the specific activity of the inorganic phosphate.

Time of incubation after tipping side arm contents (min.)	Phosphate of phosphopyruvate present		Phosphate of phosphopyruvate removed (μg.)	Phosphate of 2- and 3-phosphoglycerates present	
	Radioactive (μg.)	Non-radioactive (μg.)		Radioactive (μg.)	Non-radioactive (μg.)
0	2·3	92·7	0	5·0	0
2	2·3	88·1	4·6	—	—
4	3·1	82·5	9·4	—	—
6	3·2	77·4	14·4	—	—
8	3·4	77·0	14·6	4·8	—
10	3·5	63·8	27·7	5·4	—
15	3·5	58·1	33·4	5·1	—
20	7·1	54·3	33·6	6·8	—
30	10·6	32·9	51·5	9·2	—
60	16·6	14·8	63·6	16·4	47·2

than that of the inorganic phosphate and must therefore contain some non-isotopic phosphate. This result indicates that the formation of 2- and 3-phosphoglycerates from phosphopyruvate is reversible.

Identification of 2- and 3-phosphoglycerates. In the solvent system used (*p*-toluenesulphonic acid–*tert.*amyl alcohol–water) the substance formed from phosphopyruvate and referred to as 2- and 3-phosphoglycerates runs in the same area as authentic specimens of 2-phosphoglyceric or 3-phosphoglyceric acids. No solvent system so far tested has consistently separated the two phosphoglyceric acids. On a few occasions when a separation was achieved the substance formed from phosphopyruvate has been found in the area of 3-phosphoglyceric acid.

Formation of malate and fumarate from phosphopyruvate or 3-phosphoglyceric acid. The work of Utter & Kurahashi (1954) has demonstrated that the formation of phosphopyruvate from oxaloacetate is reversible. Table 12 demonstrates that phosphopyruvate or 3-phosphoglyceric acid can give rise to malate when incubated with pigeon-liver cyclophorase under anaerobic conditions. The amounts synthesized were small and were probably limited by the absence of suitable reducing systems in the washed homogenate. A small increase in the yield of malate was obtained by the addition of an artificial reducing system (dithionite) to the mixture. In this system very little malate is formed unless ADP is also added (Table 13).

Effect of fluoride on the interconversion of malate, phosphopyruvate and 3-phosphoglyceric acid. Phosphopyruvate was not formed from oxaloacetate under anaerobic conditions in the absence of fluoride. This is probably due to the fact that ATP required as phosphorylating agent is broken down by adenosinetriphosphatases in the absence of fluoride.

Fluoride (0·04 M) reduced the oxygen uptake in the presence of malate by about 50 % (Table 14). The formation of phosphopyruvate and of 2- and 3-phosphoglycerates is reduced to an even greater degree (Table 14, Expt. 4). Moreover, the proportion of 2- and 3-phosphoglycerates to phosphopyruvate is reduced by the presence of fluoride. This is to be expected if 2- and 3-phosphoglycerates are formed from phosphopyruvate by the action of enolase (Table 14).

Table 10. *Formation of 2- and 3-phosphoglycerates from phosphopyruvate by rat-liver mitochondria*

Rat-liver mitochondria incubated in Warburg vessels at 20° in O_2. The side arms of the vessels contained about 5 μmoles of phosphopyruvate in 0·5 ml. water. The main compartments contained 2 ml. of rat-liver mitochondria in 0·9 % (w/v) KCl, 50 μmoles citrate, 40 μmoles of phosphate buffer, pH 7·4, containing tracer amounts of $^{32}PO_4$, 4 μmoles of Na adenosine triphosphate and 4 μmoles of $MgCl_2$. Final volume 4 ml. The centre well contained 0·2 ml. of 2 N-NaOH and filter paper. The vessels were incubated for 20 min. before the contents of the side arms were tipped. One vessel was immediately removed and the amount and specific activity of the 2- and 3-phosphoglycerates was measured. The incubation of the remaining vessels was terminated at the times given below.

Time of incubation after adding phosphopyruvate (min.)	Amount of 2- and 3-phosphoglycerates present (μmoles)	Specific activity of 2- and 3-phosphoglycerates (counts/min./μg. P)
0	0·77	230
5	0·85	215
10	0·95	203
25	1·42	122
35	2·20	76
55	3·24	52

Table 11. *Formation of 2- and 3-phosphoglycerates from phosphopyruvate in pigeon-liver cyclophorase*

Pigeon-liver cyclophorase (about 80 mg. dry wt.) incubated in Warburg vessels 1 hr. in O_2 at 20°. The 4 ml. of incubation medium contained 2 ml. of the tissue suspension in 0·9 % (w/v) KCl, 2 ml. of a solution containing K phosphate buffer pH 7·4 (about 40 μmoles), Na adenosine triphosphate (about 4 μmoles), $MgCl_2$ (4 μmoles) and malate (42·3 μmoles) and the addition shown below. Tracer amounts of $^{32}PO_4$ were added. The centre well contained 0·2 ml. 2 N-NaOH and filter paper. The phosphate esters were separated on the paper chromatogram for determination of their phosphate content and of their radioactivity.

		Phosphopyruvate present			2- and 3-Phosphoglycerates present		
Expt. no.	Addition	Specific activity (counts/ min./μg. P)	Radio-active (μmoles)	Non-radio-active (μmoles)	Specific activity (counts/ min./μg. P)	Radio-active (μmoles)	Non-radio-active (μmoles)
1	Phosphopyruvate (4 μmoles)	1825	9·52	1·38	1610	3·71	1·14
	3-Phosphoglyceric acid (8 μmoles)	1990	9·70	0·50	960	4·34	5·16
2	Phosphopyruvate (5·75 μmoles)	390	4·06	0·76	392	7·88	1·47

Phosphopyruvate disappears on anaerobic incubation with pigeon-liver cyclophorase in the presence of ATP, and this disappearance is slightly inhibited by fluoride (Table 12). The removal is probably due to phosphopyruvic kinase and the fluoride effect can also be explained by an inhibition of ATPases. 3-Phosphoglyceric acid disappears on incubation with pigeon-liver cyclophorase in the absence of fluoride. In the presence of fluoride, however, added 3-phosphoglyceric acid does not disappear (see Table 12). This is again in accordance with the assumption that the interconversion of phosphopyruvate and 3-phosphoglyceric acid in the cyclophorase preparations involves enolase.

Table 12. *Formation of malate by pigeon-liver cyclophorase from phosphopyruvate or 3-phosphoglyceric acid*

Pigeon-liver cyclophorase (about 70 mg. dry wt.) incubated in Warburg vessels at 20° in N_2 with phosphorus in the centre well. The 4 ml. of incubation mixture contained 2 ml. of the tissue suspension in 0·9% (w/v) KCl and 2 ml. of a solution containing K_2 phosphate buffer pH 7·4 (0·02 M), Na adenosine triphosphate (0·002 M), $MgCl_2$ (0·002 M) and the substrate given below. Fluoride, when added, 0·0375 M: Expts. 1 and 2 incubated 30 min.; Expt. 3, 60 min.

Expt. no.	Substrate added	Amount of substrate (μmoles)	Other additions	Malate formed (μmoles)	Phospho-pyruvate present after incubation (μmoles)	2- and 3-Phospho-glycerates present after incubation (μmoles)
1	Phosphopyruvate	5·75	—	1·33	0	0·43
	Phosphopyruvate	5·75	Fluoride	1·21	0·55	0·33
	3-Phosphoglyceric acid	14·0	—	1·14	1·19	7·05
	3-Phosphoglyceric acid	14·0	Fluoride	0	0	14·0
2	3-Phosphoglyceric acid	28·0	—	1·33	—	—
	3-Phosphoglyceric acid	28·0	Fluoride	0·81	—	—
3	3-Phosphoglyceric acid	20·0	—	1·46	—	—
	3-Phosphoglyceric acid	20·0	Fluoride	0·71	—	—

Table 13. *Formation of malate from phosphopyruvate in the presence of dithionite*

Pigeon-liver cyclophorase (about 80 mg. dry wt.) incubated in Warburg vessels at 20° in N_2 with phosphorus in the centre well. The 4 ml. of incubation mixture contained 2 ml. of the tissue suspension in 0·9% (w/v) KCl and 2 ml. of a solution containing K phosphate buffer pH 7·4 (0·02 M), $MgCl_2$ (0·002 M), dithionite (30 μmoles) phosphopyruvate (6 μmoles) and adenosine diphosphate, when added, 15 μmoles.

Addition	Incubation time (min.)	Phosphopyruvate recovered (μmoles)	2- and 3-Phosphoglycerates formed (μmoles)	Malate formed (μmoles)
None	10	4·73	0·86	0
None	30	3·83	1·26	0·6
ADP	10	1·90	0·23	1·47
ADP	30	0·84	0·21	2·26

Table 14. *Effect of fluoride on oxygen consumption and phosphate esterification with malate as substrate*

Pigeon-liver cyclophorase incubated in Warburg vessels at 20° in O_2. The 4 ml. of incubation medium contained 2 ml. of the tissue suspension in 0·9% (w/v) KCl and 2 ml. of a solution containing K phosphate buffer pH 7·4 (0·2 M), Na adenosine triphosphate (0·002 M), $MgCl_2$ (0·002 M) and 42·3 μmoles of malate. Fluoride, when added, 0·0375 M final conc.; phosphopyruvate, when added, 10 μmoles. Expts. 1–3 contained about 70 mg. and Expt. 4 about 100 mg. dry wt. of cyclophorase.

			O_2 uptake		Malate removed		Phosphopyruvate found		2- and 3-Phosphoglycerates formed	
Expt. no.	Addition	Incubation time (min.)	Without fluoride (μmoles)	With fluoride (μmoles)	Without fluoride (μmoles)	With fluoride (μmoles)	Without fluoride (μmoles)	With fluoride (μmoles)	Without fluoride (μmoles)	With fluoride (μmoles)
1	Phosphopyruvate	20	5·2	1·0	—	—	—	—	—	—
2	None	40	9·5	4·7	—	—	—	—	—	—
	Phosphopyruvate	40	7·2	4·0	—	—	—	—	—	—
3	None	90	16·5	10·9	—	—	—	—	—	—
	Phosphopyruvate	90	21·0	8·9	—	—	—	—	—	—
4	None	90	30·4	13·8	22·8	16·6	4·5	0·67	8·63	0·69
	Phosphopyruvate	90	30·8	15·1	23·2	15·1	4·82	1·45	9·35	0·84

DISCUSSION

Utter & Kurahashi (1954) have demonstrated the reactions

$$\text{phosphopyruvate} + CO_2 + \text{ADP} \rightleftharpoons \text{oxaloacetate} + \text{ATP}$$

in isolated enzyme systems. Their findings satisfactorily account for the anaerobic formation of phosphopyruvate from oxaloacetate described in this paper. It remains to be explained why the aerobic rate of phosphopyruvate formation is greater from malate than from oxaloacetate. The lower reaction rate of oxaloacetate cannot be explained by an inhibitory effect of oxaloacetate since high concentrations of oxaloacetate do not inhibit phosphopyruvate formation. Moreover, it cannot be due to competition between oxidation and phosphorylation for the available oxaloacetate because, although a continuous regeneration of ATP is necessary for continued phosphopyruvate formation, the high concentration (0·0125 M) of oxaloacetate present should be enough to saturate both enzyme systems.

The observations can be explained on the basis of two assumptions; first, that the substance phosphorylated is an oxaloacetate–enzyme complex and not free oxaloacetate; secondly, that the rate of formation of this complex from oxaloacetate is slow compared with the rate of its formation from malate. Moreover, if the attachment of oxaloacetate to the enzyme involved the ketone group, it would be understandable why ketone-binding agents have so little effect on phosphopyruvate formation from malate whilst completely inhibiting its formation from oxaloacetate.

The observations that phosphopyruvate and 2- or 3-phosphoglyceric acids are interconvertible in washed liver homogenates and that this interconversion is inhibited by fluoride imply the presence of enolase within the liver particles, and thus some of the reactions of glycolysis can occur here as well as in the extra-particulate sites of the cell. Enolase has been found in the nuclei of rat liver by Dounce (1950) and it is possible that the enolase activity in the pigeon-liver preparations is also due to the presence of nuclei.

SUMMARY

1. Respiring 'cyclophorase' preparations of sheep kidney, rat liver or pigeon liver form phosphopyruvate from citrate, α-oxoglutarate, succinate, fumarate or malate. The most active tissue was pigeon liver and the highest yields were obtained from fumarate or malate.

2. Under anaerobic conditions phosphopyruvate was formed from oxaloacetate, but not from malate or fumarate; under aerobic conditions malate and fumarate formed phosphopyruvate more rapidly than oxaloacetate.

3. The formation of phosphopyruvate from malate was not abolished by semicarbazide (0·08 M), hydroxylamine (0·005 M) or cyanide (0·01 M). The major part of the malate used under these conditions was recovered as phosphopyruvate. Phosphopyruvate was not formed from oxaloacetate under these conditions. The interpretation of these facts is discussed.

4. Phosphomalate does not incorporate $^{32}PO_4$ in preparations forming phosphopyruvate and thus is not an intermediate in phosphopyruvate formation from malate.

5. Phosphate esters other than phosphopyruvate accumulate on prolonged incubation; these esters behave chromatographically like 2- and 3-phosphoglyceric acids. The formation of the esters is accounted for by the presence of a fluoride-sensitive enolase in the cyclophorase preparation.

6. Under anaerobic conditions the cyclophorase preparations form small quantities of malate from phosphopyruvate or 3-phosphoglyceric acid.

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Thermodynamic Quantities for the Dissociation Equilibria of Biologically Important Compounds

4. THE SECOND ACID DISSOCIATION OF GLUCOSE 1-PHOSPHORIC ACID

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The acid dissociations of glucose 1-phosphoric acid are important because of its key position at the beginning of the glycolysis cycle. The phosphorylase equilibria have been studied by Trevelyan, Mann & Harrison (1952) at 30°, and any extension of this work to other temperatures will require further data on the acid dissociations. Further, glucose 1-phosphoric acid falls in the intermediate range of free energies of hydrolysis, having nearly twice as large a free energy of hydrolysis as glycerol 2-phosphoric acid. It was therefore of interest to see whether the increased energy of hydrolysis was reflected in an altered dissociation constant or in the alteration of any of the thermodynamic quantities of ionization. Glucose 1-phosphoric acid may be used as a buffer and as such has certain advantages over phosphoric acid. More extensive data on the system are therefore desirable, in particular for standardization purposes.

Only the second dissociation constant has been measured. The prolonged equilibration periods of the cells and the low first pK, coupled with the acid lability of glucose 1-phosphoric acid, render the first dissociation constant inaccessible to the present technique.

EXPERIMENTAL

Glucose 1-phosphoric acid. The preparation was based on McCready & Hassid's (1944) modification of Hanes's (1940) method, but with considerable modifications in detail which were found essential in order to obtain good yields of material of high purity. Three solutions were prepared: (*a*) 100 g. soluble starch suspended in 500 ml. water poured slowly into 3·5 l. boiling water and boiled for 2 min. Much less viscous and more even suspensions were obtained with soluble starch than with potato starch. (*b*) A buffer solution pH 6·8, by dissolving 1 mole NaH_2PO_4, $2H_2O$ (136 g.) and 1 mole Na_2HPO_4, $12H_2O$ (358 g.) in 3·0 l. water. (*c*) Potato extract, 1100 ml., made by scraping 6 lb. of potatoes, mincing finely and expressing the juice into 100 ml. 0·01 M-KCN (neutralized), allowing it to settle and decanting. The cyanide is essential to prevent the formation of quinones, which discolour the final product. No significant effect of the age of the potatoes was observed. The three solutions were mixed, 50 ml. of toluene added, and the final mixture was incubated at 25° for 24 hr. At the end of the incubation, the mixture was boiled to precipitate the proteins and, without filtering, a solution of Mg acetate added (85 g. MgO in 500 ml. water and 250 ml. acetic acid), followed by ammonia (sp.gr. 0·88) to pH 8·4. The mixture was placed in the cold room for 48 hr. and the combined precipitates of protein and inorganic phosphate were filtered off. The precipitation of inorganic phosphate is not complete until 48 hr. and if the period of standing in the cold is reduced, it contaminates the final product.

The glucose 1-phosphate was separated from acetate, excess starch, etc., by means of ion exchange. The solution was passed through a 'Zeocarb 215' cation-exchange resin column of bed volume 1080 ml. at the rate of 5 bed vol./hr. and at once through an 'Amberlite IR-4B' anion-exchange column saturated with acetate (see below) at the rate of 8 bed vol./hr. The bed volume of this column was 690 ml. It is essential that there should be no delay once the cations are removed, since the solution is then sufficiently acid to cause slow hydrolysis of the glucose 1-phosphoric acid and this leads to contamination of the final product with inorganic phosphate. With the reagent concentrations used here, the 'Zeocarb 215' becomes saturated after approx. 3 bed vol. have passed through. It is preferable to allow most of the effluent to flow directly into the anion exchanger, testing only the last portions to find when the cation exchanger needs regeneration. Similarly, it is preferable to arrange the bed volumes so that the anion exchanger does not require regeneration during the course of a run. The ester is then removed in a single batch, yields are improved and contamination lessened. When all the ester was attached to the anion exchanger, the column was washed with 3–4 l. water to remove soluble impurities, starch and dextrins. The ester was then eluted by passing M-KOH at 3 bed vol./hr. through the column. McCready & Hassid (1944) recommend ammonia for the elution, but no advantage is apparent in its use. KOH gave sharper limits for the elution and yielded the K salt directly, thus eliminating the necessity for boiling off the ammonia with KOH. Resinous decomposition products were thereby avoided. Acetate is first removed from the column and then the greater part of the ester follows in the next 500 ml. of effluent. This solution was treated with 50 g. K acetate and 10% (w/v) KOH to pH 12·0. The solution was heated to 70–80° and 1·3 vol. of boiling methanol were added. Dipotassium glucose 1-phosphate, $2H_2O$ crystallized at once. The solution was allowed to stand overnight in the refrigerator, and filtered, the crystals washed with methanol and ether and dried *in vacuo* over $CaCl_2$. The yield was generally 40 g. The reduction of the volume of the methanol from the 1·5 vol. recommended by McCready & Hassid slightly reduces the yield, but has the advantage of giving crystals

Table 1. *Glucose 1-phosphoric acid; second dissociation. Molalities (m) and ionic strength (I) of solutions, e.m.f.'s of cells (E) in absolute volts (corrected to 1 atm. pressure of hydrogen and rounded temperatures) and extrapolation functions (y)*

m_1 = molality of HCl, m_2 = molality of dipotassium glucose 1-phosphate, $I = 3m_2 - m_1$ (one value for I suffices for all temperatures, since m_{H^+} is negligible)

$$y = \frac{(E - E_0)\,F}{2{\cdot}3026\,RT} + \log \frac{m_1^2}{m_2 - m_1} + \frac{2AI^{\frac{1}{2}}}{1 + I^{\frac{1}{2}}}.$$

Two sets of values appear for 25° in set 1, on one day the values from 5 to 25° were determined, on the next day the values from 25 to 50° were determined on the same dilutions. In sets 2 and 3 the e.m.f.'s were determined with no break in the measurements.

Set 1

	$10^2\,m_1$	0·28107	0·45021	0·54891	0·68049	0·81899	1·14029	1·36864
	$10^2\,m_2$	0·53352	0·85460	1·04195	1·29173	1·55462	2·16450	2·59860
	$10^2\,I$	1·31950	2·11359	2·57694	3·19470	3·84487	5·35321	6·42716
5°	E	0·72593	0·71338	0·70792	0·70244	0·69737	0·68841	0·68343
	y	6·51283	6·51351	6·51186	6·51899	6·51968	6·52462	6·52755
10°	E	0·73177	0·71907	0·71359	0·70782	0·70270	0·69357	0·68850
	y	6·50756	6·50971	6·50954	6·51336	6·51489	6·51986	6·52288
15°	E	0·73760	0·72478	0·71911	0·71320	0·70802	0·69872	0·69354
	y	6·50447	6·50863	6·50692	6·51019	6·51234	6·51737	6·52017
20°	E	0·74351	0·73038	0·72470	0·71860	0·71339	0·70391	0·69871
	y	6·50598	6·50885	6·50877	6·51064	6·51394	6·51885	6·52298
25°	E	0·74938	0·73601	0·73027	0·72401	0·71874	0·70909	0·70375
	y	6·50968	6·51248	6·51312	6·51418	6·51807	6·52305	6·52644
	E	0·74933	—	0·73017	0·72409	0·71866	0·70908	0·70367
	y	6·50884	—	6·51143	6·51553	6·51671	6·52289	6·52508
30°	E	0·75526	0·74166	0·73565	0·72947	0·72401	0·71418	0·70868
	y	6·51585	6·51879	6·51665	6·52091	6·52324	6·52814	6·53049
35°	E	0·76117	0·74721	0·74123	0·73487	0·72936	0·71933	0·71374
	y	6·52599	6·52695	6·52704	6·53015	6·53325	6·53778	6·54029
40°	E	0·76713	0·75291	0·74677	0·74028	0·73472	0·72455	0·71894
	y	6·53880	6·53947	6·53866	6·54149	6·54535	6·55050	6·55429
45°	E	0·77306	0·75856	0·75235	0·74564	0·74002	0·72976	0·72399
	y	6·55348	6·55357	6·55333	6·55445	6·55890	6·56546	6·56829
50°	E	0·77899	0·76418	0·75804	0·75106	0·74543	0·73498	0·72910
	y	6·56855	6·56763	6·57014	6·56884	6·57465	6·58103	6·58371

Set 2

	$10^2\,m_1$	0·48654	0·57212	0·73609	0·78686	0·90469	1·02023	1·35337	1·48090	1·90750	2·11031	2·23824
	$10^2\,m_2$	0·91999	1·08178	1·39192	1·48788	1·71066	1·92941	2·55917	2·80035	3·60750	3·99093	4·23324
	$10^2\,I$	2·26003	2·65746	3·41979	3·65574	4·20132	4·73823	6·28417	6·87970	8·85750	9·80279	10·39772
25°	E	0·73911	0·73555	0·72900	0·72715	0·72301	0·71911	0·70888	0·70500	0·69450	0·68966	0·68677
	y	6·51284	6·51284	6·51284	6·51284	6·51284	6·51284	6·51284	6·51284	6·51284	6·51284	6·51284
30°	E	0·74504	0·74144	0·73484	0·73297	0·72880	0·72486	0·71452	0·71059	0·69997	0·69508	0·69216
	y	6·52124	6·52124	6·52124	6·52124	6·52124	6·52124	6·52124	6·52124	6·52124	6·52124	6·52124
35°	E	0·75102	0·74738	0·74073	0·73884	0·73464	0·73066	0·72024	0·71627	0·70554	0·70060	0·69765
	y	6·53000	6·53000	6·53000	6·53000	6·53000	6·53000	6·53000	6·53000	6·53000	6·53000	6·53000
40°	E	0·75705	0·75337	0·74667	0·74477	0·74053	0·73651	0·72601	0·72200	0·71116	0·70617	0·70319
	y	6·53911	6·53911	6·53911	6·53911	6·53911	6·53911	6·53911	6·53911	6·53911	6·53911	6·53911
45°	E	0·76312	0·75940	0·75265	0·75073	0·74646	0·74240	0·73181	0·72776	0·71681	0·71178	0·70877
	y	6·54857	6·54857	6·54857	6·54857	6·54857	6·54857	6·54857	6·54857	6·54857	6·54857	6·54857
50°	E	0·76923	0·76548	0·75867	0·75673	0·75243	0·74833	0·73765	0·73356	0·72250	0·71742	0·71439
	y	6·55838	6·55838	6·55838	6·55838	6·55838	6·55838	6·55838	6·55838	6·55838	6·55838	6·55838

Set 2

	$10^2\,m_1$	0·48654	0·57212	0·73609	0·78686	0·90469	1·02023	1·35337	1·48090	1·90750	2·11031	2·23824
	$10^2\,m_2$	0·72556	0·85318	1·09770	1·17340	1·34912	1·52143	2·01822	2·20839	2·84457	3·14700	3·33779
	$10^2\,I$	1·69014	1·98742	2·55701	2·73334	3·14268	3·54406	4·70128	5·14427	6·62621	7·33070	7·77513
5°	E	0·69736	0·69315	0·68646	0·68463	0·68075	0·67752	0·66991	0·66761	0·66055	0·65776	0·65616
	y	6·50729	6·50973	6·51202	6·51172	6·51063	6·51203	6·51628	6·52030	6·52184	6·52338	6·52483
10°	E	0·70283	0·69835	0·69158	0·68976	0·68594	0·68255	0·67478	0·67237	0·66522	0·66238	0·66077
	y	6·50461	6·50365	6·50678	6·50745	6·50856	6·50819	6·51220	6·51505	6·51739	6·51900	6·52083
15°	E	0·70828	0·70366	0·69675	0·69488	0·69106	0·68746	0·67963	0·67709	0·66987	0·66696	0·66532
	y	6·50389	6·50195	6·50485	6·50506	6·50759	6·50467	6·51021	6·51157	6·51507	6·51640	6·51824
20°	E	0·71366	0·70896	0·70191	0·70001	0·69617	0·69244	0·68448	0·68186	0·67456	0·67161	0·66993
	y	6·50515	6·50327	6·50596	6·50625	6·50966	6·50565	6·51145	6·51226	6·51671	6·51830	6·51998
25°	E	0·71907	0·71426	0·70708	0·70513	0·70125	0·69745	0·68933	0·68662	0·67931	0·67625	0·67454
	y	6·50977	6·50748	6·51011	6·51014	6·51406	6·51001	6·51557	6·51568	6·52225	6·52289	6·52461
30°	E	0·72442	0·71956	0·71221	0·71025	0·70633	0·70246	0·69414	0·69140	0·68396	0·68087	0·67912
	y	6·51520	6·51400	6·51595	6·51639	6·52082	6·51674	6·52143	6·52186	6·52849	6·52958	6·53114
35°	E	0·72987	0·72493	0·71743	0·71534	0·71141	0·70747	0·69902	0·69619	0·68866	0·68551	0·68374
	y	6·52677	6·52514	6·52676	6·52565	6·53105	6·52696	6·53198	6·53173	6·53910	6·54011	6·54187
40°	E	0·73530	0·73028	0·72259	0·72034	0·71653	0·71253	0·70382	0·70093	0·69334	0·69009	0·68831
	y	6·53934	6·53781	6·53850	6·53539	6·54385	6·53989	6·54313	6·54273	6·55130	6·55162	6·55374
45°	E	0·74072	0·73561	0·72777	0·72540	0·72161	0·71749	0·70859	0·70566	0·69794	0·69472	0·69288
	y	6·55412	6·55253	6·55292	6·54854	6·55836	6·55361	6·55625	6·55603	6·56468	6·56639	6·56806
50°	E	0·74616	0·74096	0·73290	0·73065	0·72676	0·72254	0·71315	0·71055	0·70269	0·69940	0·69755
	y	6·56962	6·56797	6·56701	6·56513	6·57442	6·56920	6·56661	6·57233	6·58091	6·58240	6·58443

Set 3

	$10^2\,m_1$	0·45790	0·56729	0·65144	0·72797	0·76903	0·88568	1·24728	1·38777	1·61826	1·80308	2·11431	2·19992
	$10^2\,m_2$	0·64252	0·79602	0·91410	1·02148	1·07911	1·24278	1·75018	1·94732	2·27074	2·53008	2·96679	3·08693
	$10^2\,I$	1·46967	1·82077	2·09085	2·33648	2·46828	2·84266	4·00325	4·45420	5·19397	5·78716	6·78607	7·06086
5°	E	0·69452	0·68898	0·68529	0·68225	0·68078	0·67691	0·66774	0·66476	0·66063	0·65781	0·65330	0·65221
	y	6·50843	6·51168	6·51219	6·51150	6·51184	6·51141	6·51602	6·51579	6·51883	6·52288	6·52285	6·52356
10°	E	0·69987	0·69420	0·69037	0·68729	0·68581	0·68190	0·67254	0·66957	0·66533	0·66245	0·65791	0·65671
	y	6·50448	6·50726	6·50652	6·50614	6·50680	6·50695	6·51129	6·51225	6·51474	6·51869	6·51968	6·51880
15°	E	0·70513	0·69935	0·69547	0·69233	0·69085	0·68687	0·67734	0·67432	0·66999	0·66707	0·66244	0·66119
	y	6·50130	6·50401	6·50364	6·50321	6·50435	6·50456	6·50901	6·51007	6·51240	6·51661	6·51753	6·51618
20°	E	0·71045	0·70453	0·70061	0·69743	0·69590	0·69185	0·68217	0·67906	0·67466	0·67171	0·66695	0·66570
	y	6·50240	6·50452	6·50468	6·50457	6·50532	6·50559	6·51050	6·51097	6·51349	6·51814	6·51832	6·51736
25°	E	0·71581	0·70975	0·70579	0·70252	0·70099	0·69686	0·68703	0·68385	0·67938	0·67638	0·67150	0·67025
	y	6·50704	6·50860	6·50928	6·50861	6·50984	6·51000	6·51536	6·51562	6·51832	6·52305	6·52268	6·52212
30°	E	0·72113	0·71493	0·71097	0·70762	0·70606	0·70186	0·69186	0·68863	0·68408	0·68102	0·67604	0·67483
	y	6·51332	6·51434	6·51620	6·51517	6·51635	6·51659	6·52208	6·52246	6·52519	6·52984	6·52929	6·52977
35°	E	0·72646	0·72013	0·71613	0·71277	0·71113	0·70692	0·69672	0·69344	0·68883	0·68568	0·68067	0·67939
	y	6·52323	6·52391	6·52626	6·52603	6·52635	6·52764	6·53279	6·53330	6·53639	6·54047	6·54090	6·54059
40°	E	0·73181	0·72536	0·72126	0·71784	0·71622	0·71195	0·70155	0·69823	0·69352	0·69034	0·68525	0·68391
	y	6·53534	6·53585	6·53772	6·53747	6·53858	6·54009	6·54491	6·54572	6·54853	6·55303	6·55362	6·55272
45°	E	0·73719	0·73061	0·72641	0·72299	0·72130	0·71701	0·70643	0·70304	0·69828	0·69501	0·68985	0·68851
	y	6·55029	6·55050	6·55189	6·55258	6·55302	6·55539	6·56023	6·56085	6·56417	6·56815	6·56905	6·56853
50°	E	0·74260	0·73584	0·73160	0·72812	0·72641	0·72207	0·71128	0·70786	0·70298	0·69969	0·69442	0·69302
	y	6·56611	6·56524	6·56713	6·56779	6·56837	6·57109	6·57551	6·57658	6·57932	6·58388	6·58447	6·58339

which are free from the brown material eluted from the resin by the alkali. Any ester remaining in solution can be recovered by including the mother liquors in the next batch of filtrate which is put through the columns. The presence of the methanol appears not to have any deleterious effect upon the performance of the resins. Ester intended for potentiometric work was recrystallized 3 times by dissolving 40 g. in 500 ml. water and adding methanol as before.

The cation-exchange columns were 5 cm. in diameter and those for the anion exchanger 7 cm. in diameter. The large cross-section of the anion exchanger has two advantages: first, the acid solution has only a short distance to travel before encountering fresh resin, so that the time during which hydrolysis of the ester could occur is minimized; secondly, the swelling of the Amberlite IR-4B which follows its absorption of anions was less likely to burst the wide column. Troubles with swelling of the resin were finally overcome by passing 5% (w/v) acetic acid upwards through the column after it had been prepared for use followed by the removal of the excess acid with distilled water. The resin was then fully expanded and the displacement of acetate by glucose 1-phosphate was effected with but little volume change. This technique has the added advantage of freeing the column from CO_2 which may otherwise be evolved during the absorption of the ester, with disruption of the column and reduced efficiency of absorption.

The 'Zeocarb 215' was prepared for use by passing 4% (w/v) HCl through the column at 6 bed vol./hr. until the pH of the effluent fell below pH 1. This took approximately 2 l. of acid; the resin was then washed free of acid with dist. water at 10 bed vol./hr. until the pH rose to that of the water. The 'Amberlite IR-4B' was generated by passing 5% (w/v) KOH at 4 bed vol./hr., until the effluent was at pH 11–12. The resin was then washed free of alkali with dist. water at 10 bed vol./hr. until the pH dropped to near 7. If the resin is to be used in the acetate form it is unnecessary to wash so thoroughly before introducing the acetic acid.

After the passage of the solution through the cation exchanger, it contains only free acids and its pH is near 1. The exhaustion of the resin is indicated by a sudden sharp rise in the pH of the effluent to near 4. The solution in the column was then displaced with 1 bed vol. of water and the column regenerated. The elution of the ester from the columns was followed by boiling a few drops of the effluent with acid molybdate at intervals.

The purity of the potassium glucose 1-phosphate could not be assayed by incineration to $K_4P_2O_7$ as was possible with sodium glycerol 2-phosphate. The melting point of the potassium salt was low and the decomposing melt entrained carbon, which it was subsequently impossible to incinerate. Instead, the salt was assayed by weight titrations with standard HCl to an end point at pH 3·4 (glass electrode). Glucose (by Benedict's method after hydrolysis) and the specific rotation were also determined. The rotation found was higher than any recorded in the literature. Typical analytical figures were: weight titration, 99·98–100·06% pure; glucose, expected 48·4%, found 48·1%; $[\alpha]_D^{25}$, literature value; 78°; found: 79·7°. Chromatography in a *tert.*-amyl alcohol–water–*p*-toluene sulphonic acid solvent (Hanes & Isherwood, 1949) showed only a single spot. Controls indicated that less than 1% of inorganic phosphate or other esters could be detected.

There is thus good evidence that the material was highly purified $C_6H_{11}O_6.PO_3K_2$, $2H_2O$. It is usually assumed that the ester prepared by phosphorylase has the α-configuration and that assumption is followed here.

Potentiometry. This was carried out using the same apparatus and technique as for glycerol 2-phosphoric acid (Ashby, Crook & Datta, 1954*a*). Three sets of experiments were done on different batches of glucose 1-phosphoric acid, the results being combined as before by averaging the coefficients of the orthogonal polynomials used to fit the thermodynamic pK values to the temperature curve. During the early part of this investigation it became necessary, owing to leakage of paraffin from the thermostat bath into the platinum resistance thermometer, to obtain a new thermometer from the Cambridge Instrument Company. It was subsequently discovered that the calibration of this thermometer was incorrect. A more suitable platinum resistance thermometer was therefore constructed according to the design of Barber (1950), and this was calibrated by the National Physical Laboratory. At the same time the two standard resistances and the two standard cells were also checked by the National Physical Laboratory. The resistance of the thermometer at the triple point of water (273·17° K) was rechecked frequently during the course of this work using a triple-point cell (Barber, Handley & Herington, 1954).

RESULTS

The measurements and calculations of the three sets of experiments are summarized in the tables. Table 1 gives the molalities of the solutions, the e.m.f. measurements and the calculated extrapolation functions for each of the three sets of dilutions. Table 2 contains the values of the thermodynamic pK values obtained from the least squares extrapolations of the results in Table 1, together with the calculated values of the pK values from the equation

$$pK = A/T - D + CT,$$

the constants of this equation, and the minimum pK and the temperature at which it occurs. The slopes of the regression lines 'β' are also included as they will be of value to those wishing to calculate the pH of glucose 1-phosphate–chloride buffers. Table 3 contains the thermodynamic quantities of the ionization calculated from the constants of the above equation, together with the standard errors of the results, calculated according to Please (1954).

DISCUSSION

The present results lend weight to the previous conclusion that the methods of Harned and his collaborators are applicable to phosphate esters of biological importance. The errors found in this work are of the same order of magnitude as for glycerol 2-phosphoric acid (Ashby, Crook & Datta, 1954*b*), and the wider experience now obtained suggests that the chief source of variance lies in the difficulty of preparing the esters in a sufficiently high degree of purity, especially as regards water content of the

Table 2. *Glucose 1-phosphoric acid; second dissociation. Observed and calculated values of* pK_2 *at rounded temperatures, the values of 'β' the slope of the extrapolation, the constants A, C and D of the equation* $pK_2 = A/T - D + CT$, *where T is the absolute temperature, the minimum value of* pK_2 *and the temperature at which it occurs*

The constants of the equation $pK_2 = A/T - D + CT$ were obtained by the method of least squares and the calculated values of pK_2 were obtained by using this equation.

The values of pK_2 observed are given to four decimal places, as these figures were used to calculate the constants of the equation relating pK_2 to temperature.

Temp. (°)	Set 1		Set 2		Set 3		
	pK (obs.)	β	pK (obs.)	β	pK (obs.)	β	pK (calc.)
5	6·5070	0·32	6·5036	0·28	6·5055	0·26	6·506
10	6·5029	0·31	6·4995	0·27	6·5004	0·28	6·500
15	6·5006	0·31	6·4982	0·25	6·4973	0·29	6·499
20	6·5010	0·34	6·4992	0·26	6·4983	0·30	6·500
25	6·5045	0·33	6·5032	0·27	6·5025	0·30	6·504
30	6·5113	0·30	6·5092	0·28	6·5086	0·32	6·510
35	6·5207	0·31	6·5198	0·27	6·5184	0·34	6·519
37	—	—	—	—	—	—	6·524
40	6·5322	0·33	6·5320	0·27	6·5301	0·35	6·531
45	6·5464	0·33	6·5462	0·26	6·5445	0·37	6·545
50	6·5613	0·34	6·5611	0·27	6·5599	0·37	6·561

$\sigma = \pm 3 \times 10^{-4}$

$A = 1432{\cdot}1569$; $D = 3{\cdot}4213$; $C = 0{\cdot}017177$; $pK_{min.} = 6{\cdot}498$; $T_{min.} = 15{\cdot}6°$.

Table 3. *Glucose 1-phosphoric acid; second dissociation. Thermodynamic quantities*

Temp. (°)	$\Delta G°$		$\Delta H°$		$-\Delta S°$		$-\Delta C_p°$	
	Cal.	Joule	Cal.	Joule	Cal. deg.$^{-1}$	Joule deg.$^{-1}$	Cal. deg.$^{-1}$	Joule deg.$^{-1}$
5	8277	34 626	474	1980	28·1	117·4	43·7	183
10	8419	35 221	254	1060	28·85	120·7	44·5	186
15	8566	35 832	29	120	29·6	124·0	45·3	189
20	8716	36 460	−199	−830	30·4	127·3	46·1	193
25	8870	37 105	−431	−1800	31·2	130·5	46·8	196
30	9028	37 765	−668	−2790	32·0	133·8	47·6	199
35	9190	38 443	−908	−3800	32·8	137·1	48·4	203
37	9255	38 718	−1005	−4200	33·1	138·4	48·7	204
40	9355	39 136	−1152	−4820	33·6	140·4	49·2	206
45	9525	39 847	−1400	−5860	34·35	143·7	50·0	209
50	9699	40 573	−1652	−6910	35·1	147·0	50·8	212
	Errors							
	σ_5, σ_{25}, σ_{50}, = standard errors at 5, 25 and 50°, respectively							
σ_5	0·7	3·1	21·7	91	0·08	0·32	0·90	3·8
σ_{25}	0·45	1·9	6·3	26	0·02	0·09	0·97	4·1
σ_{50}	0·7	3·1	24·0	100	0·08	0·32	1·05	4·4

Table 4. *Thermodynamic* pK_2 *values of orthophosphoric acid and ester phosphates at* 30°

Acid	pK_2	Author
Orthophosphoric	7·190	Bates & Acree (1943)
Glycerol 2-phosphoric	6·657	Ashby, Crook & Datta (1954*b*)
Glucose 1-phosphoric	6·510	This paper
Glucose 1-phosphoric	6·51 ± 0·01	Trevelyan, Mann & Harrison (1952)
Glucose 1-phosphoric	6·47*	Cori, Colowick & Cori (1937)

* Calculated from the apparent pK_2 using the Guntelberg approximation for the activity coefficients (Ashby, Crook & Datta, 1954*a*).

crystals. Thus a difference in the water of crystallization of 2 parts in 1000 between batches would produce errors in pK of the same order of magnitude as those actually observed. In view of this, the close agreement between batches must be regarded as very satisfactory. With glucose 1-phosphoric acid the most important source of error to be suspected is that due to hydrolysis. Tests showed that under the conditions here used no hydrolysis could be detected during the course of a set of measurements.

No values of the thermodynamic quantities are available in the literature for comparison, but, as with glycerol 2-phosphoric acid, these fall within the range of Harned & Owen's (1950) data. Comparison with glycerol 2-phosphoric acid shows the relation to be complex, but the most striking difference is the larger value of $-\Delta C_p^\circ$ for glucose 1-phosphoric acid. The minimum pK and the temperature at which they occur are similar for the two esters.

The only value of the thermodynamic pK_2 for glucose 1-phosphoric acid in the literature is that obtained by Trevelyan *et al.* (1952) at 30°, by the extrapolation of glass electrode measurements to infinite dilution. An apparent pK_2 has also been determined by Cori, Colowick & Cori (1937). This value has been recalculated to an approximate thermodynamic value by use of the Guntelberg approximation for the activity coefficients (Ashby *et al.* 1954*a*). The results at 30°, together with the thermodynamic pK_2 values for glycerol 2-phosphoric acid and orthophosphoric acid, are shown in Table 4. The agreement between the present results and those of Trevelyan *et al.* is very good indeed, and that with the results of Cori *et al.* is reasonably close considering the inaccuracy of correcting, without extrapolation for the effects of ionic strength on the activity coefficients.

In view of the greater standard free energy of hydrolysis, it is interesting that the second acid dissociation constant of glucose 1-phosphoric acid is stronger than that of glycerol 2-phosphoric acid by approximately 0·15 pK unit.

SUMMARY

1. The second thermodynamic dissociation constant of glucose 1-phosphoric acid has been measured at 5° intervals from 5 to 50° using hydrogen and silver–silver chloride electrodes in cells without liquid junction.

2. From these measurements have been calculated the standard free energy, heat content, entropy and heat capacity for the ionization over the whole temperature range, together with their standard deviations.

3. The data presented allow the use of glucose 1-phosphoric acid as a standard buffer.

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Thermodynamic Quantities for the Dissociation Equilibria of Biologically Important Compounds

5. THE SECOND ACID DISSOCIATION OF 2-AMINOETHANOL 1-PHOSPHORIC ACID

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2-Aminoethanol 1-phosphoric acid is widely distributed in animal tissues both in the free state (Walker, 1952) and in combination (Campbell & Work, 1952); it may be synthesized easily (Outhouse, 1937) and thus lends itself to study as a model for other compounds containing both ester phosphate and amino groups, e.g. creatine phosphate.

THEORY

From the pH titration curves of 2-aminoethanol 1-phosphoric acid with NaOH and HCl, and from the formol titration (Figs. 1 and 2) it is seen that the three acid dissociations are widely separated and that the dissociation near pH 10 may be attributed to the amino group. From these considerations, and by analogy with taurine (2-aminoethanol 1-sulphonic acid; King, 1953), the three acid-base equilibria may be represented as shown below, the free ester being a dipolar ion (II).

It will be convenient to refer to these various species as R^+, R^{+-}, R^{+2-} and R^{2-} respectively.

K_{2a} has been determined on the molal scale in cells without liquid junction of the type:

Pt, H_2 (1 atm.) | 2-aminoethanol 1-phosphoric acid (m_1), NaOH (m_2), NaCl (m_3) | AgCl, Ag

at 5° intervals from 5 to 50°.

The e.m.f. of this cell is given by

$$E = E_0 - \frac{RT}{F} \ln a_{H^+} . a_{Cl^-} . \qquad (1)$$

From this equation, in terms of molalities, and using the Guntelberg approximation for the activity coefficients (Ashby, Crook & Datta, 1954*a*) we get

$$-\log m_{H^+} = \frac{(E - E_0)\,F}{2{\cdot}3026RT} + \log m_{Cl^-} - \frac{2AI^{\frac{1}{2}}}{1 + I^{\frac{1}{2}}} \qquad (2)$$

the linear term in I being neglected.

The dissociation constant under consideration is

$$K_{2a} = \frac{a_{H^+} . a_{R^{+-}}}{a_{R^{+-}}} . \qquad (3)$$

Combining eqns. 1 and 3 and eliminating m_{H^+} we get

$$-\log K_{2a} = \frac{(E - E_0)\,F}{2{\cdot}3026RT} + \log \frac{m_{Cl^-} . m_{R^{+-}}}{m_{R^{+2-}}} + \log \frac{\gamma_{Cl^-} . \gamma_{R^{+-}}}{\gamma_{R^{+2-}}} . \qquad (4)$$

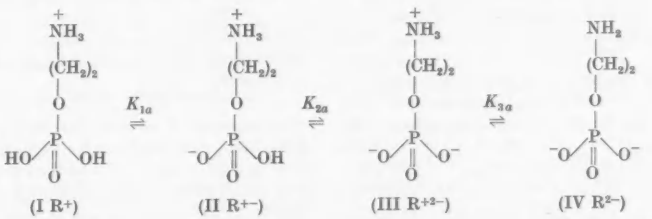
The dipolar ion R^{+-} may be considered as an uncharged molecule with unit activity coefficient, and the ion R^{+2-} may be considered as carrying a unit negative charge. Thus the activity coefficient term in eqn. 4, when approximated as before, reduces to a linear term in I, and eqn. 4 becomes

$$-\log K_{2a} + \beta I = \frac{(E - E_0)\,F}{2{\cdot}3026RT} + \log \frac{m_{Cl^-} . m_{R^{+-}}}{m_{R^{+2-}}} = y, \qquad (5)$$

where $m_{R^{+-}} = m_1 - m_2 - m_{H^+}$, $m_{R^{+2-}} = m_2 + m_{H^+}$ and $m_{Cl^-} = m_3$; and

$$I = m_2 + m_3 + m_{H^+} . \qquad (6)$$

In the solutions studied m_{H^+} was so small that the value at one temperature only was evaluated and used in all calculations.



(I R^+) (II R^{+-}) (III R^{+2-}) (IV R^{2-})

The right-hand side (y) of eqn. 5 is plotted against I to give a straight line of slope 'β'; the intercept at $I=0$, giving $-\log K_{2a}$, is obtained by the method of least squares.

The values of $-\log K_{2a}$ at different temperatures were found to fit the Harned & Robinson (1940) equation

$$-\log K_{2a} = A/T - D + CT. \qquad (7)$$

The constants of this equation were calculated for each set of dilutions and their standard deviations calculated by the methods previously described (Please, 1954).

The values of pK_{1a}, pK_{2a} and pK_{3a} on the molar scale were estimated by pH titration curve measurement, with a glass electrode at 20°. They were obtained from the titration curves by the well-known equation

$$pK = pH + \log \frac{a_{\text{acid species}}}{a_{\text{base species}}}.$$

This equation, in terms of molarities and with the same approximation for the activity coefficients, becomes for K_{1a}, K_{2a} and K_{3a} respectively:

$$pK_{1a} = pH + \log \frac{C_{R^+}}{C_{R^{+-}}} - \frac{AI^{\frac{1}{2}}}{1+I^{\frac{1}{2}}} - \beta_1 I, \qquad (8)$$

$$pK_{2a} = pH + \log \frac{C_{R^{+-}}}{C_{R^{+2-}}} + \frac{AI^{\frac{1}{2}}}{1+I^{\frac{1}{2}}} - \beta_2 I, \qquad (9)$$

$$pK_{3a} = pH + \log \frac{C_{R^{+2-}}}{C_{R^{2-}}} + \frac{3AI^{\frac{1}{2}}}{1+I^{\frac{1}{2}}} - \beta_3 I. \qquad (10)$$

The values of the coefficients β_1, β_2 and β_3 cannot be determined from a single titration curve, and as they are usually small they may be neglected to a first approximation.

Fundamental constants. The values of E_0 for the silver–silver chloride electrode of Harned & Ehlers (1933) were used as before (Ashby *et al.* 1954*a*) but

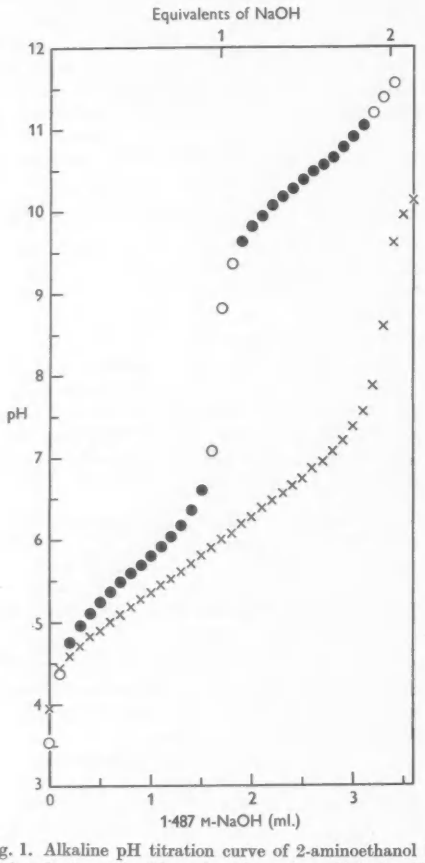


Fig. 1. Alkaline pH titration curve of 2-aminoethanol 1-phosphoric acid with and without formaldehyde, 20°. ○○●●, 25 ml. 0·1 M 2-aminoethanol 1-phosphoric acid +25 ml. 0·3 M-NaCl; × × × ×, 25 ml. 0·1 M 2-aminoethanol 1-phosphoric acid +15 ml. 40% (w/v) formaldehyde +10 ml. water. The full points were used to calculate pK_{2a} and pK_{3a}.

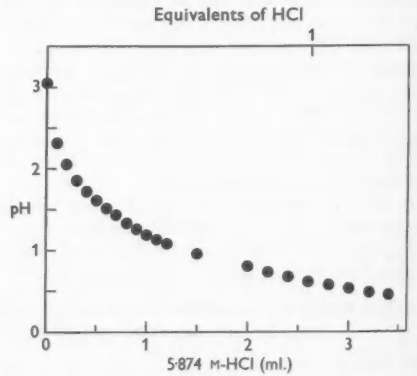


Fig. 2. Acid pH titration curve of 2-aminoethanol 1-phosphoric acid, 20°. ●●●●, 2·1723 g. 2-aminoethanol 1-phosphoric acid in 20 ml. water.

Table 1. *Values of fundamental constants used in this work*

$R = 8{\cdot}31436$ joules (absolute) deg.$^{-1}$ mol.$^{-1}$ (chemical scale).

$T = 273{\cdot}16 + t°$ C.

$F = 96493{\cdot}9$ coulombs (absolute) g.mol.$^{-1}$ (chemical scale).

1 cal. (defined) $= 4{\cdot}1840$ joules (absolute).

The values of R and F are from the values given by DuMond & Cohen (1953) on the physical scale divided by 1·000272. The values of T and the ratio J/cal. are those given by Rossini, Gucker, Johnston, Pauling & Vinal (1952).

were converted into absolute volts by multiplying by the factor 1·00033 (DuMond & Cohen, 1948). All e.m.f. measurements and calculations were done in absolute volts. The most recent values of other constants were used; these are listed in Table 1.

METHODS

The apparatus and general technique for the measurements in cells without liquid junction have been described previously (Ashby *et al.* 1954*a*). The measurement of temperature, the preparation of carbonate-free NaOH and standard HCl, the glass electrode standardization and measurements have also been described (Clarke, Cusworth & Datta, 1954).

Preparation of 2-aminoethanol 1-phosphoric acid. The ester was prepared by allowing $POCl_3$ to react with aqueous 2-aminoethanol as described by Outhouse (1937). The reaction mixture was diluted with *ca.* 20 vol. water and passed through columns of 'Deacidite FF' (The Permutit Co. Ltd., London, W. 4), a strongly basic anion-exchange resin, in the hydroxyl phase. The columns were then thoroughly washed with boiled-out distilled water to remove free 2-aminoethanol. The 2-aminoethanol 1-phosphoric acid was then displaced from the resin with M-HCl. The ester came off first, followed by phosphoric acid and HCl. The pH of the liquid containing the ester was between 4 and 2. Fractions were tested for reaction with ninhydrin, for inorganic and ester phosphate with acid molybdate, and for chloride. Those fractions giving both positive reactions with ninhydrin and for bound phosphate, but no reaction for inorganic phosphate or chloride, were combined and evaporated to dryness under reduced pressure. The residue, which was slightly coloured, was dissolved in a small amount of distilled water, boiled with decolorizing charcoal and filtered. The clear filtrate was then treated with 4 vol. methanol. The free ester crystallized almost immediately, and more came out of solution on standing overnight in the refrigerator. The crystals were filtered off, washed with methanol and dried *in vacuo* over P_2O_5. The ester was twice reprecipitated with methanol from water and finally recrystallized from conductivity water.

Typical analytical results were as follows: m.p. (corr.) 243·5–244·5°; 243·0–244·0° (Outhouse, 1937, 244°); inorganic P: 0·04 and 0·00% of total P; total P: 99·73 and 100·40% of required amount; total N: 99·3, 99·7, 99·97, 99·90 and 100·1% of required amount. Chromatography showed only one ninhydrin-reacting spot.

The ester was considered to be highly purified 2-aminoethanol 1-phosphoric acid. There was no water of crystallization.

Sodium chloride (AnalaR) was recrystallized once from water with HCl gas and ignited. No free acid could be detected in the salt.

Formaldehyde for the formol titration was shaken with heavy magnesium carbonate and the titration was carried out according to the method of Dunn & Loshakoff (1936).

RESULTS

The molalities and ionic strengths of the solutions, the e.m.f.'s of the cells and the extrapolation function y (eqn. 5) of the solutions examined in cells without liquid junction are shown in Table 2. The extrapolated values of $-\log K_{2a}$ (pK_{2a} (obs.)) at infinite dilution, the slopes 'β' of the extrapolations and the values of the ratio $m_{R^+-}/m_{R^{+2}-}$ at 25° are given in Table 3. The values of pK_{2a} (calc.) were derived from eqn. 7, the constants of which were obtained by averaging the coefficients of the orthogonal polynomials used in fitting the curves relating pK_{2a} (obs.) to temperature for each set of results. There was no significant difference between the three sets. The values of the constants of eqn. 7 and the minimum value of pK_{2a} together with the temperature at which it occurs are also given. The thermodynamic quantities associated with the second acid dissociation are given in Table 4.

The mean values of pK_{1a}, pK_{2a} and pK_{3a} on the molar scale, obtained from points on the pH titration curves (Figs. 1 and 2) and eqns. 8–10, neglecting the linear term in I, are given in Table 5. The number of points on the pH titration curves used in the calculations, together with the standard errors, are also given.

The value of pK_{1a} is given as <1 because the values drifted downwards from 0·68 to 0·30 as the titration with HCl proceeded. This pK may be considered so low as to be inaccessible to measurement by this method with a glass electrode. The solutions must of necessity be strongly acid, and the corrections in the molarity terms due to the hydrogen-ion concentration must be large.

For pK_{2a}, values for β_2 are available, being very nearly the same as for 'β' in eqn. 5; the difference between the molar and the molal scales is negligible at 20°. Values of 'β_2' for the appropriate buffer ratios $m_{R^+-}/m_{R^{+2}-}$ in the titration curve were found from Fig. 3. The mean value of pK_{2a} thus corrected is also given in Table 5; it may be seen that the standard deviation is slightly reduced by this correction. For comparison the value of pK_{2a} at 20° from cells without liquid junction, converted to the molar scale by applying the correction given in Ashby *et al.* (1954*a*; Table 2, p. 195), is also given. The corrected value of pK_{2a} from the pH titration curve is seen to be very close to that obtained from cells without liquid junction, the difference being within the tolerance of both the pH scale and the primary standard of pH (British Standards Institution (1950) 1647).

In Table 6 are collected the pK values of 2-aminoethanol 1-phosphoric acid, some other phosphoric acid esters, orthophosphoric acid, taurine and 2-aminoethanol.

DISCUSSION

The results given in Table 3 show that the assumptions about the activity coefficients of the various ionic species and their contributions to the ionic strength are justified. If these assumptions were seriously incorrect, the values of pK_{2a} (obs.)

Table 2. *2-Aminoethanol 1-phosphoric acid. Molalities (m) and ionic strengths (I) of solutions, e.m.f.'s of cells (E), absolute volts (corrected to 1 atm. pressure of hydrogen and rounded temperatures) and extrapolation functions (y)*

m_1 = molality of 2-aminoethanol 1-phosphoric acid; m_2 = molality NaOH; m_3 = molality NaCl;
m_{H^+} = antilog $[-(E-E_0)\ F/2{\cdot}3026RT - \log m_{Cl^-} + 2AI^{\frac{1}{2}}/(1+I^{\frac{1}{2}})]$; $I = m_2 + m_3 + m_{H^+}$;
$y = -\log K_{2a} + \beta I = (E-E_0)\ F/2{\cdot}3026RT + \log m_{Cl^-}.m_{R^{+-}}/m_{R^{+\pm}}$.

Set 1

$10^2\ m_1$		0·79277	1·98223	3·17152	4·36069	5·54959	6·73850	7·92719	9·08142
$10^2\ m_2$		0·39979	0·99963	1·59938	2·19906	2·79863	3·39819	3·99764	4·57971
$10^2\ m_3$		0·39988	0·99986	1·59976	2·19958	2·79928	3·39899	3·99857	4·58078
$10^6\ m_{H^+}$	(25°)	2	2	2	2	2	2	3	3
$10^2\ I$		0·79985	1·99969	3·19935	4·39886	5·59814	6·79742	7·99646	9·16075
5°	*E*	0·68867	0·66637	0·65501	0·64707	0·64109	0·63629	0·63226	0·62880
	y	5·83276	5·82689	5·82522	5·81966	5·81602	5·81338	5·81090	5·80724
10°	*E*	0·69393	0·67128	0·65968	0·65161	0·64554	0·64068	0·63659	0·63306
	y	5·82824	5·82328	5·82097	5·81564	5·81231	5·81013	5·80788	5·80408
15°	*E*	0·69924	0·67618	0·66439	0·65620	0·65001	0·64505	0·64089	0·63731
	y	5·82707	5·82193	5·81989	5·81494	5·81139	5·80897	5·80675	5·80316
20°	*E*	0·70454	0·68112	0·66909	0·66078	0·65448	0·64948	0·64524	0·64161
	y	5·82868	5·82423	5·82158	5·81701	5·81342	5·81178	5·80943	5·80605
25°	*E*	0·70991	0·68604	0·67383	0·66538	0·65900	0·65386	0·64955	0·64584
	y	5·83407	5·82877	5·82654	5·82201	5·81888	5·81632	5·81401	5·81032
30°	*E*	0·71523	0·69095	0·67854	0·66994	0·66348	0·65824	0·65386	0·65007
	y	5·84115	5·83569	5·83354	5·82887	5·82619	5·82340	5·82113	5·81714
35°	*E*	0·72047	0·69585	0·68317	0·67445	0·66791	0·66260	0·65812	0·65434
	y	5·85016	5·84568	5·84247	5·83815	5·83590	5·83338	5·83066	5·82786
40°	*E*	0·72583	0·70071	0·68787	0·67900	0·67231	0·66693	0·66232	0·65853
	y	5·86301	5·85691	5·85443	5·84998	5·84703	5·84477	5·84112	5·83915
45°	*E*	0·73113	0·70558	0·69245	0·68352	0·67655	0·67128	0·66652	0·66270
	y	5·87708	5·87053	5·86670	5·86354	5·85784	5·85869	5·85383	5·85234
50°	*E*	0·73633	0·71041	0·69690	0·68801	0·68097	0·67564	0·67072	0·66669
	y	5·88993	5·88386	5·87733	5·87698	5·87190	5·87310	5·86691	5·86309

Set 2

$10^2\ m_1$		0·90217	1·80448	2·70681	3·60914	4·51145	5·41375	6·31613	7·21843
$10^2\ m_2$		0·57349	1·14707	1·72066	2·29426	2·86784	3·44141	4·01503	4·58861
$10^2\ m_3$		0·62629	1·25268	1·87908	2·50549	3·13187	3·75825	4·38469	5·01107
$10^6\ m_{H^+}$	(35°)	1	1	1	1	1·5	2	2	2
$10^2\ I$		1·19989	2·39987	3·59987	4·79989	5·99986	7·19982	8·39989	9·59986
5°	*E*	0·69118	0·67418	0·66472	0·65767	0·65209	0·64758	0·64376	0·64054
	y	5·83894	5·83206	5·83679	5·83398	5·82980	5·82728	5·82502	5·82468
10°	*E*	0·69652	0·67923	0·66957	0·66238	0·65674	0·65215	0·64825	0·64499
	y	5·83504	5·82844	5·83264	5·82960	5·82613	5·82363	5·82117	5·82114
15°	*E*	0·70187	0·68429	0·67446	0·66711	0·66139	0·65671	0·65277	0·64943
	y	5·83377	5·82744	5·83165	5·82802	5·82490	5·82225	5·82029	5·81987
20°	*E*	0·70724	0·68940	0·67933	0·67186	0·66608	0·66131	0·65732	0·65391
	y	5·83580	5·83024	5·83325	5·82976	5·82731	5·82450	5·82286	5·82224
25°	*E*	0·71263	0·69449	0·68421	0·67658	0·67075	0·66590	0·66185	0·65838
	y	5·84075	5·83526	5·83763	5·83359	5·83196	5·82918	5·82767	5·82701
30°	*E*	0·71798	0·69955	0·68902	0·68125	0·67538	0·67042	0·66633	0·66282
	y	5·84757	5·84232	5·84340	5·83916	5·83849	5·83523	5·83419	5·83383
35°	*E*	0·72336	0·70461	0·69383	0·68591	0·68007	0·67498	0·67082	0·66727
	y	5·85813	5·85261	5·85244	5·84784	5·84925	5·84520	5·84412	5·84405
40°	*E*	0·72869	0·70966	0·69854	0·69045	0·68468	0·67951	0·67526	0·67168
	y	5·86974	5·86461	5·86179	5·85652	5·86058	5·85657	5·85513	5·85551
45°	*E*	0·73401	0·71466	0·70326	0·69492	0·68922	0·68405	0·67975	0·67608
	y	5·88340	5·87802	5·87357	5·86639	5·87302	5·87032	5·86916	5·86902
50°	*E*	0·73928	0·71950	0·70805	0·69982	0·69366	0·68852	0·68413	0·68037
	y	5·89664	5·88929	5·88685	5·88343	5·88429	5·88332	5·88181	5·88116

Table 2 (*cont.*)

Set 3

$10^2\ m_1$		1·18513	2·76632	5·92856	7·50974	9·09089	10·67212	12·25329
$10^2\ m_2$		0·31072	0·72528	1·55435	1·96891	2·38346	2·79802	3·21258
$10^2\ m_3$		0·58868	1·37410	2·94486	3·73027	4·51567	5·30110	6·08651
$10^6\ m_{H^+}$	(35°)	5	5·5	6	7	7	7	8
$10^2\ I$		0·89988	2·09991	4·49983	5·69984	6·89983	8·09985	9·29984
5°	*E*	0·65420	0·63337	0·61426	0·60824	0·60334	0·59914	0·59551
	y	5·83239	5·82355	5·80855	5·80216	5·79638	5·78994	5·78418
10°	*E*	0·65889	0·63766	0·61825	0·61214	0·60715	0·60287	0·59919
	y	5·82875	5·81947	5·80524	5·79917	5·79336	5·78684	5·78135
15°	*E*	0·66359	0·64196	0·62225	0·61604	0·61096	0·60663	0·60287
	y	5·82774	5·81801	5·80452	5·79859	5·79275	5·78668	5·78092
20°	*E*	0·66831	0·64628	0·62628	0·61997	0·61478	0·61039	0·60657
	y	5·83000	5·81985	5·80726	5·80147	5·79524	5·78944	5·78377
25°	*E*	0·67301	0·65061	0·63030	0·62388	0·61859	0·61413	0·61025
	y	5·83452	5·82447	5·81240	5·80657	5·80015	5·79443	5·78885
30°	*E*	0·67770	0·65492	0·63430	0·62780	0·62237	0·61789	0·61389
	y	5·84142	5·83129	5·81974	5·81436	5·80709	5·80228	5·79578
35°	*E*	0·68234	0·65919	0·63825	0·63169	0·62612	0·62159	0·61749
	y	5·85073	5·84070	5·82947	5·82486	5·81677	5·81235	5·80530
40°	*E*	0·68704	0·66344	0·64217	0·63552	0·62986	0·62536	0·62099
	y	5·86291	5·85169	5·84062	5·83628	5·82819	5·82543	5·81511
45°	*E*	0·69168	0·66765	0·64600	0·63930	0·63347	0·62890	0·62449
	y	5·87634	5·86427	5·85256	5·84911	5·83976	5·83703	5·82718
50°	*E*	0·69628	0·67185	0·64960	0·64298	0·63709	0·63259	0·62806
	y	5·88950	5·87708	5·86132	5·86075	5·85190	5·85138	5·84074

Table 3. *2-Aminoethanol 1-phosphoric acid, second dissociation. Observed and calculated values of* pK_{2a} *at rounded temperatures, together with '*β*' the slopes of the extrapolations,* $m_{R^+-}/m_{R^{+2}-}$ *the buffer ratios, the constants of the equation* $pK_{2a} = A/T - D + CT$, *the minimum* pK_{2a} *and the temperature at which it occurs*

The calculated values of pK_{2a} are from the equation relating pK_{2a} to temperature, the standard deviation, σ, was calculated according to Please (1954). Values of pK_{2a} (obs.) are given to four decimal places, as these values were used to calculated A, C, and D and pK_2 (calc.).

	Set 1		Set 2		Set 3		
Temp. (°)	pK_{2a} (obs.)	$-'\beta'$	pK_{2a} (obs.)	$-'\beta'$	pK_{2a} (obs.)	$-'\beta'$	pK_{2a} (calc.)
5	5·8338	0·296	5·8401	0·166	5·8358	0·569	5·836
10	5·8293	0·281	5·8360	0·163	5·8319	0·557	5·832
15	5·8282	0·278	5·8348	0·163	5·8305	0·545	5·832
20	5·8297	0·264	5·8368	0·159	5·8326	0·536	5·834
25	5·8350	0·272	5·8415	0·160	5·8372	0·530	5·838
30	5·8420	0·273	5·8480	0·161	5·8441	0·525	5·845
35	5·8510	0·259	5·8578	0·160	5·8536	0·519	5·854
37	—	—	—	—	—	—	5·858
40	5·8634	0·277	5·8686	0·157	5·8653	0·525	5·865
45	5·8768	0·285	5·8808	0·147	5·8785	0·542	5·878
50	5·8898	0·289	5·8942	0·154	5·8901	0·529	5·893
$\frac{m_{R^+-}}{m_{R^{+2}-}}$ (25°)		0·98		0·57		2·81	$\sigma = \pm 6 \times 10^4$

$A = 1228{\cdot}3378$; $C = 0{\cdot}01493$; $D = 2{\cdot}7328$; $pK_{min.} = 5{\cdot}832$; $t_{min.} = 13{\cdot}7°$.

Table 4. *2-Aminoethanol 1-phosphoric acid; second dissociation. Thermodynamic quantities*

Temp. (°)	$\Delta G°$ Joule	$\Delta G°$ Cal.	$\Delta H°$ Joule	$\Delta H°$ Cal.	$-\Delta S°$ Joule deg.$^{-1}$	$-\Delta S°$ Cal. deg.$^{-1}$	$-\Delta C_p°$ Joule deg.$^{-1}$	$-\Delta C_p°$ Cal. deg.$^{-1}$
5	31 077	7428	1402	335	106·6	25·5	159·0	38·0
10	31 617	7557	600	143	109·5	26·2	161·8	38·7
15	32 172	7689	−217	−52	112·4	26·9	164·7	39·7
20	32 741	7825	−1047	−250	115·2	27·5	167·6	40·1
25	33 325	7965	−1892	−452	118·1	28·2	170·4	40·7
30	33 923	8108	−2752	−658	120·9	28·9	173·3	41·4
35	34 535	8254	−3625	−866	123·8	29·6	176·1	42·1
37	34 783	8313	−3979	−951	124·9	29·9	177·3	42·4
40	35 161	8404	−4513	−1079	126·7	30·3	179·1	42·8
45	35 801	8557	−5415	−1294	129·5	31·0	181·9	43·5
50	36 456	8713	−6332	−1513	132·4	31·6	184·7	44·1
				Errors				
				σ_5, σ_{25}, σ_{50} = standard errors at 5, 25 and 50° respectively.				
σ_5	3·7	0·9	112	27	0·39	0·09	4·6	1·1
σ_{25}	2·2	0·5	34	8	0·11	0·03	5·0	1·2
σ_{50}	3·7	0·9	123	29	0·39	0·09	5·4	1·3

Table 5. *2-Aminoethanol 1-phosphoric acid. pK_{1a}, pK_{2a} and pK_{3a} at 20° on the molar scale*

These values were obtained from the pH titration curves (glass electrode) shown in Figs. 1 and 2. The number of points used in the calculations and the standard errors are also given.

$$pK_{1a} = pH + \log C_R{}^{+}/C_R{}^{+-} - AI^{\frac{1}{2}}/(1+I^{\frac{1}{2}});$$
$$pK_{2a} = pH + \log C_R{}^{+-}/C_R{}^{\pm 2-} + AI^{\frac{1}{2}}/(1+I^{\frac{1}{2}});$$
$$pK^{*}_{2a} = pK_{2a} - \beta_2 I;\ \beta_2 \text{ being found from Fig. 3;}$$
$$pK_{3a} = pH + \log C_R{}^{\pm 2-}/C_R{}^{2-} + 3AI^{\frac{1}{2}}/(1+I^{\frac{1}{2}});$$

pK^{*}_{2a} was found from cells without liquid junction corrected to the molar scale.

$\sigma\lambda$	pK	No. of points used	σ
pK_{1a}	<1	—	—
pK_{2a}	5·783	14	±0·021
pK^{*}_{2a}	5·831	14	±0·016
$pK^{\dagger}_{2a}$	5·835	—	±0·0006
pK_{3a}	10·89	13	±0·014

obtained at very different buffer ratios would not show such good agreement. As the differences between the three sets of results are not statistically significant, it may be assumed that they are mostly due to variations in the ester and errors in the experimental technique. It is, however, of interest to note that the slopes 'β' of the extrapolations vary markedly with the buffer ratio, indicating that there is some residual systematic effect on the activity coefficients of one or other of the ions which is not accounted for by the approximation used. Such marked variation of 'β' with the buffer ratio was not seen with the other phosphate esters which have been studied. The usefulness of the Guntelberg approximation for the activity coefficients, together with a linear term in I, in correcting results from glass electrode measurements of the pH titration curve to give the thermodynamic pK is well illustrated by the results in Table 5.

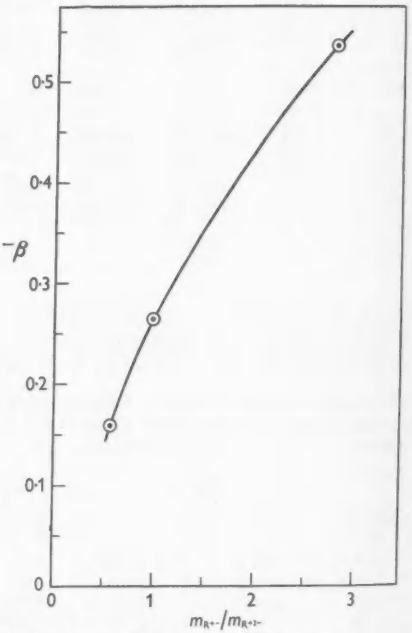


Fig. 3. Variation of the slopes 'β' of the extrapolations of the results from cells without liquid junction with the buffer ratio $m_{R^+}/m_{R^{\pm}2-}$. The curve is from data given in Table 3.

The errors in the determination of both pK_{2a} and the thermodynamic quantities are similar to those found in the determinations of pK_{2a} of glycerol 2-phosphoric acid and glucose 1-phosphoric acid. This is surprising, since, in the present experiments,

Table 6. *The acid dissociation constants of 2-aminoethanol 1-phosphoric acid and a number of related compounds*

Substance	pK_{1a} (20°)	M	A	pK_{2a} (25°)	M	A	$pK_{NH_3^+}$ (20°)	M	A
2-Aminoethanol 1-phosphoric acid	<1	1	1	5·838	2	1	10·89	1	1
Glucose 1-phosphoric acid	1·23 (30°)	1	3	6·504	2	4	—	—	—
Glycerol 2-phosphoric acid	1·301	2	2	6·650	2	2	—	—	—
H_3PO_4	2·127	2	5	7·198	2	6	—	—	—
2-Aminoethanol	—	—	—	—	—	—	9·47 (25°)	3	8
Taurine	—	—	—	—	—	—	9·186	2	7

M = method

1 Glass electrode with liquid junction
2 Cells without liquid junction
3 H_2 electrode with liquid junction

A = author

1 This paper
2 Ashby, Crook & Datta (1954*b*)
3 Cori, Colowick & Cori (1937)
4 Ashby, Clarke, Crook & Datta (1955)
5 Bates (1951)
6 Bates & Acree (1943)
7 King (1953)
8 Simms (1928)

the solutions were made up with NaOH with the attendant errors in standardization, freedom from carbon dioxide contamination, etc.

From the results given in Table 6 it is seen that both the first and second acid dissociations of 2-aminoethanol 1-phosphoric acid are markedly stronger than those of the sugar phosphoric acids, which are themselves stronger than those of orthophosphoric acid. This increased strength of the two phosphate hydrogen dissociations over those of the sugar phosphates is presumably due to the powerful inductive effect of the positive charge on the nitrogen atom (cf. Kumler & Eiler, 1943). It is to be noted that the $pK_{NH_3^+}$ of 2-aminoethanol 1-phosphoric acid is considerably higher than that of taurine, again presumably due to the inductive effect of the two negative charges on the phosphate oxygens in place of the single negative charge on the sulphonic oxygens. This increased charge would be expected to impede the dissociation of the amino hydrogen.

The thermodynamic quantities associated with the second acid dissociation of 2-aminoethanol 1-phosphoric acid are similar to those for other ester phosphates, as is the temperature at which the maximum dissociation occurs.

SUMMARY

1. The second thermodynamic dissociation constant of 2-aminoethanol 1-phosphoric acid on the molal scale has been measured at 5° intervals from 5 to 50° using hydrogen and silver–silver chloride electrodes in cells without liquid junction.

2. From these measurements have been calculated the standard free energy, heat content, entropy and heat capacity for the ionization over the whole temperature range.

3. The first, second and third dissociation constants of 2-aminoethanol 1-phosphoric acid on the molar scale have been estimated at 20° from glass-electrode measurements with liquid junction.

4. It is demonstrated how the activity coefficients may be allowed for in the glass-electrode measurements.

5. The marked differences between the acid dissociation constants of 2-aminoethanol 1-phosphoric acid and those of similar acids are discussed.

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Toxicity of Aromatic Acids to the larvae of the Mosquito *Aedes aegypti* L. and the Counteracting Influence of Amino Acids

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Knowledge of the detoxication mechanisms of insects is becoming of ever-increasing importance. The metabolism of insecticides may so alter their biological activity as to destroy their toxicity (Sternburg & Kearns, 1950), produce new poisons in the plant from applied chemicals (Casida, Chapman, Stahmann & Allen, 1954; Metcalf, 1954) or even form the material which is toxic to insects from a non-toxic precursor (Metcalf & March, 1953; Casida & Stahmann, 1953). Locusts have been reported to contain the enzymes for glucuronide and ethereal sulphate hydrolysis (Robinson, Smith & Williams, 1953); they also form hippuric acid from benzoic acid (Friedler & Smith, 1953), glucosides from phenols (Myers & Smith, 1953*a*, 1954) and can acetylate aromatic amines (Myers & Smith, 1953*b*). In addition, insects can dehydrochlorinate certain chlorinated hydrocarbons enzymically (Sternburg, Vinson & Kearns, 1953), and oxidize thiophosphates (Metcalf & March, 1953) and dimethylphosphoramides (Casida, Allen & Stahmann, 1953). However, attempts to demonstrate methylation in *Bombyx mori* and *Lucilia caesar* by the isolation of *N'*-methylnicotinamide following the feeding of nicotinamide have proved unsuccessful (Kato, 1953).

Aromatic and amino acids play an important role in the life of insects. Aromatic acids may serve as egg-hatching factors for mosquitoes (Abdel-Malek, 1948; Gjullin, Yates & Stage, 1939), and as insecticides or repellents (Bushland, 1940; Fennah, 1950; Swingle, Phillips & Gahan, 1944; and others). Although the toxicity of a few aromatic acids has been studied with mosquito larvae (Bodine, 1923; Bushland & King, 1943), no detailed studies on the structural relationship for aromatic acid toxicity have been reported. In regard to amino acids, isoleucine is required for oviposition by adult *Aedes aegypti* (Greenburg, 1951) and the larvae require ten amino acids for growth (Goldberg & DeMeillon, 1948). Paper chromatograms have identified twenty amino acids from adult Culicid mosquitoes (Clark & Ball, 1952) and eighteen from all the developmental stages of *Aedes aegypti* L. (Micks & Ellis, 1951, 1952).

The recent identification of a conjugation product such as hippuric acid (Friedler & Smith, 1953) in locusts does not necessarily indicate that the toxicity of benzoic acid to the insect was actually reduced by this conjugation. The ease of studying a detoxication problem with mosquito larvae bioassay prompted the use of this technique. The investigation reported here concerns the toxicity of various aromatic acids to mosquito larvae with particular reference to the counteracting influence of amino acids.

MATERIALS AND METHODS

Mosquito larvae. A small colony of *Aedes aegypti* L. was maintained (Casanges, McGovern & Chiles, 1949) as a source of eggs, which were collected on moist filter paper and dried so that they could be hatched when desired for use. The first-instar larvae generally used for assay were not fed before testing; later instars were fed on finely ground dog biscuits (Friskies Dog Food Meal) until the actual assay time, when only the experimental nutrients were provided. Larval instars were differentiated on the basis of head capsule width. The adult mosquitoes were fed 5% sucrose solutions except for a single blood meal immediately after emergence.

Compounds. The amino acids and aromatic acids tested were of the highest purity obtainable commercially. The samples of D- and L-glutamic acids were optically pure. The identities of the substituted benzoic acids were ascertained by melting points and neutralization equivalents. All amino acids were tested at 0·1 M concentration unless specifically stated otherwise. In the study of hydrogen-ion concentration, a buffer which was 0·02 M with respect to each of

acetate, orthophosphate and borate was used. All other studies utilized an 0·02M phosphate buffer, pH 7·0. The final pH of all solutions was confirmed before assay and did not change during the assay time.

Biological testing. Bioassays were made in 12 × 75 mm. tubes with 1·0 ml. total liquid volume per tube. The papers with the eggs were soaked in distilled water for about 8 hr., then the larvae were concentrated to about 20 larvae/drop by utilizing their negative phototropic response. To each assay tube was then added one drop (about 20 larvae) and the tubes kept at 27° for 24 hr., after which time the mortality was recorded. The mortality end-point was considered to be an inability of the larvae to move in response to a strong light source. Mortality comparisons were based on dosage/mortality curves with from 10–15 serial dilutions for each aromatic acid and a 1·3- to 1·6-fold dilution factor, depending on the precision desired for the results. The LD_{50} (molar concentration of toxicant effecting a 50% mortality) and confidence limits were determined by the method of Litchfield & Wilcoxon (1949) in which the probit of the percentage mortality was plotted against the logarithm of the molar concentration of toxicant. No consistent correlation of slope with relative toxicity or specific counteracting agents was evident.

The efficiency of an amino acid in these studies was considered to be its relative ability to decrease the toxicity of the aromatic acid involved. This was expressed as the 'toxicity index' which is the ratio between the LD_{50} of the aromatic acid alone and the LD_{50} in the presence of the amino acid, multiplied by 100 (Sun, 1950). Thus a figure of less than 100 would indicate that the amino acid decreased the toxicity of the aromatic acid.

Biosynthesis of hippuric acid. Three lots of about 300 fourth-instar *A. aegypti* larvae were starved for 24 hr. and then added to a solution containing 0·167M [α-^{14}C]glycine and 0·15M benzoic acid in 0·033M phosphate buffer at pH 7·0. After 36 hr. incubation at 25° the larvae were collected on filter paper, washed with distilled water and then homogenized (Potter & Elvehjem, 1936). The larval homogenate, the supernatant fluid in which the larvae had been swimming, and a sample of the glycine–benzoic acid solution comparable to that in which the larvae were originally suspended were then acidified with H_2SO_4 and extracted with a butanol–chloroform mixture according to the method of Chantrenne (1951). No [^{14}C]glycine was extracted into the organic phase, whereas a radioactive material formed by the mosquito larvae, presumably hippuric acid, appeared in this layer.

Further proof of the structure of the biosynthesized material extracted by the butanol–chloroform mixture was afforded by paper chromatography and co-chromatography with known samples of glycine and hippuric acid. For this purpose the solvent system used by Fewster & Hall (1951) for organic acids was modified to utilize the organic phase from a mixture of 60 ml. *n*-butanol, 30 ml. 95% (v/v) ethanol and 30 ml. of a 1·5N solution of ammonium carbonate and ammonia. Whatman no. 1 paper was used in upward-flow chromatography (Wolfson, Cohn & Devaney, 1949) at 25° and the glycine spot with a R_F of 0·11 was localized with ninhydrin while the hippuric acid of R_F 0·48 was detected with a pH indicator reagent of methyl red and bromophenol blue. The radioactive materials after incubation with mosquito larvae were co-chromatographed with known glycine and hippuric samples and the position and shape of the coloured spots compared with a radioautograph.

RESULTS

It was found in preliminary experiments that mosquito larvae were sensitive to aromatic acids and that this toxic action was reversed by gelatin and certain amino acids. Of twelve possible counteracting materials tested in combination with benzoic acid, all except L-cysteine and α-D-glucuronic acid decreased the toxicity of the benzoic to the mosquito larvae significantly.

Effect of larval instar. The toxicity of benzoic acid was tested against the four larval instars of *A. aegypti* (instars differentiated on the basis of head capsule width). The fourth instar was only about half as sensitive to the benzoic acid as were the three younger stages, which did not differ significantly in their susceptibility. The toxicity of benzoic acid to all instars was significantly reduced in the presence of either glycine or DL-ornithine (0·1M). The amino acids were the most efficient in reducing the toxicity of the benzoic acid with the first-instar and the least with the fourth-instar larvae, while the second and third instars did not differ significantly in this respect. Therefore, newly hatched first-instar larvae were selected as the best assay system for further study.

Influence of pH. A study of the effect of pH on the toxicity and counteraction by amino acids was made since both glycine and benzoic acid are partially ionized at pH 7·0. The toxicity of the benzoic acid increased significantly as the pH was lowered from 9·0 to 4·0. Compared with the toxicity at pH 7·0, benzoic was only two-thirds as toxic at pH 9·0, but was over 10 times as toxic at pH 5 and 20 times at pH 4·0. The counteracting influence of glycine was lost above pH 7·0, suggesting that the undissociated or cationic forms were primarily responsible for this reduction in toxicity. Further assays were standarized at pH 7·0.

Effect of amino acids. The effect of amino acids in counteracting the toxicity of benzoic acid is shown in detail in Table 1. The following relationships are evident: (1) Glycine and readily available sources of glycine such as glycylglycine and gelatin were the best counteracting agents tested. (2) The efficiency decreased with increasing chain length as shown with the glycine, alanine, valine, and leucine series, serine and threonine, and ornithine and lysine. The only discrepancy in this relationship was with glutamic acid or glutamine and aspartic acid or asparagine. (3) Serine and threonine were fairly comparable with their non-hydroxyl analogues. (4) The thio amino acids required the SH group blocked for counteraction, cf. cysteine with methionine and cystine. (5) Dicarboxylic acids were better than their amides. L-Glutamic acid was not significantly different from the D- or DL- acids. (6) The amino group is most efficient in the

Table 1. *Efficiency of various amino acids in counteraction of benzoic acid toxicity*

Two series of experiments designated as *A* and *B* are shown. In series *A* the LD_{50} for benzoic acid was 0·028 (0·025–0·031) M, while in series *B* the value was 0·023 (0·020–0·026) M. The toxicity index is the ratio between the molar LD_{50} value with benzoic acid alone and the molar LD_{50} of benzoic in the presence of 0·100 M amino acid, multiplied by 100. Each toxicity index is followed by the confidence limits for 19/20 probability. Those amino acids designated by * were tested partially in suspension, while others were in solution.

	Toxicity index
Series *A*	
Varying chain length	
Glycine	51 (45–55)
DL-Alanine	59 (55–65)
DL-Valine	56 (54–59)
DL-Isoleucine	62 (57–67)
L-Leucine	73 (68–78)
DL-Norleucine	73 (67–86)
Hydroxy amino acids	
L-Serine	57 (51–63)
DL-Threonine	65 (60–70)
Thio amino acids	
L-Cystine*	68 (62–74)
DL-Methionine	79 (74–85)
L-Cystine	120 (113–128)
Aromatic amino acids	
L-Proline	58 (54–62)
DL-Tryptophan	66 (62–70)
DL-Phenylalanine*	71 (66–76)
L-Tyrosine	79 (73–86)
L-Histidine	96 (89–102)
L-Hydroxyproline	105 (96–115)
Position of amino group	
DL-α-Alanine	59 (55–65)
β-Alanine	75 (68–82)
Availability of α-amino group	
Glycine	51 (45–55)
Glycylglycine	51 (48–55)
Betaine	118 (112–125)
Creatine	82 (74–90)
Creatinine	96 (87–106)
Series *B*	
Control	
Glycine	28 (26–30)
Isomers of glutamic acid	
L-Glutamic	42 (39–46)
D-Glutamic	45 (43–47)
DL-Glutamic	45 (41–50)
Dicarboxylic amino acids and their amides	
L-Glutamic	42 (39–46)
L-Glutamine	50 (45–56)
L-Aspartic	46 (42–51)
L-Asparagine	69 (63–77)
Basic amino acids	
DL-Arginine	39 (34–44)
DL-Ornithine	46 (42–50)
L-Lysine	51 (47–56)
DL-Citrulline	65 (58–73)

α-position (cf. α- and β-DL-alanines) and must be free as shown with betaine and glycine or glycylglycine, and with creatine and creatinine. (7) Of possible metabolic significance are the marked differences between arginine and citrulline, cystine and cysteine, and proline and hydroxyproline.

Effect of different levels of glycine, arginine and p-hydroxybenzoic acid on benzoic acid toxicity. Competition experiments were made with the two most efficient amino acids, glycine and arginine, and with the least toxic benzoic acid derivative, *p*-hydroxybenzoic acid. Fig. 1 shows this relationship.

With glycine the reduction in toxicity of benzoic acid was almost directly proportional to the logarithm of the glycine concentration up to 0·05 M, after which no further change resulted. At this optimum point in the curve, the benzoic acid and glycine were equimolar. At the three lowest concentrations the glycine appeared to reduce the toxicity of the benzoic acid as if a mole-for-mole detoxication were involved. Arginine was less effective, reached a lower optimum and was toxic itself at concentrations of 0·1 M. On the other hand, *p*-hydroxybenzoic acid increased the toxicity of benzoic acid up to 0·05 M in such a manner as to suggest a synergistic action since by itself it is non-toxic in this concentration range.

In vivo *hippuric acid synthesis by mosquito larvae.* A preliminary test demonstrated the presence of [^{14}C]hippuric acid in first-instar mosquito larvae incubated with [α-^{14}C]glycine and benzoic acid. When about 100 fourth-instar larvae/ml. were incubated for 36 hr. at 25° with 0·15 M benzoic acid

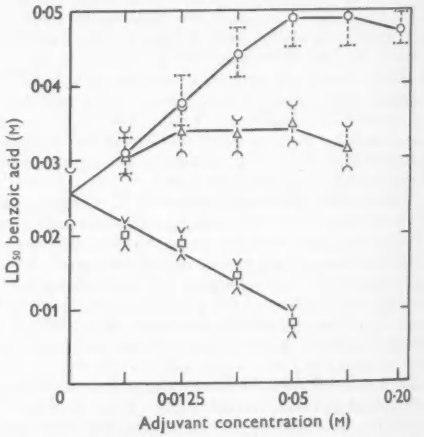

Fig. 1. Effects of glycine, arginine and *p*-hydroxybenzoic acid on the toxicity of benzoic acid. For conditions see the text. ○, glycine; △, arginine; □, *p*-hydroxybenzoic acid.

and 0·167 M [α-^{14}C]glycine, the larvae contained 12 % of the radioactivity, the remaining 88 % being in the supernatant. Of the latter radioactivity, 27·3 % was present as hippuric acid, whereas 43·5 % of the ^{14}C in the larvae was present as this acid. The identity of the biosynthesized radioactive hippuric acid was ascertained when co-chromatography with the authentic acid yielded a spot which was identical in position and shape to that found on the radioautograph of the biosynthesized material. In the radioautograph a very small amount of a faster moving component (R_F 0·70) also appeared.

These results indicate that mosquito larvae form hippuric acid *in vivo* in the presence of glycine and benzoic acid and that the conjugated derivative is probably excreted.

Relation of structure of aromatic acids to toxicity. Structural specificity studies for these aromatic acids are summarized in Table 2. The *para* substitution by halogens appeared to be the most effective and the *ortho* substitution the least in enhancing the toxicity of these aromatic acids. This was particularly evident with 2:6-dichlorobenzoic acid. Iodo derivatives were generally more toxic and displayed a different structural specificity than did the chloro derivatives. The most toxic substituted benzoic acid found was 2-hydroxy-3:5-diiodobenzoic acid, which was 5 times as toxic as 2:3:5-triiodobenzoic acid. The toxicity of 3:5-diiodobenzoic acid was increased by an *ortho*-hydroxyl or *para*-iodo group, but was decreased by an *ortho*-iodo substitution. This indicates that different combinations of ring substituents might yield still more toxic compounds. The *para*-chloro derivatives of benzoic, phenylacetic, diphenylacetic and phenoxyacetic acids were all more toxic than the parent acids. Of ten different *para* substituents, the iodo was the most toxic and the hydroxyl the least. An incomplete study indicates that the toxicity of the halogen generally increased and the glycine detoxifying efficiency decreased as the electronegative properties of the *para* halogen decreased. Side-chain variations greatly altered the activity in the phenyl series. Increasing the chain length in a normal aliphatic series decreased the toxicity. Phenoxy compounds were poorer than phenyl with either phenylacetic or diphenylacetic derivatives. Benzyl alcohol was 4 times as toxic as either benzoic acid or benzaldehyde. The sulphinic and phosphonic acid side chains were essentially inactive. Benzoic acid was 15 times as toxic as its glycine conjugate, hippuric acid. Among the ring variations, benzoic was more toxic than nicotinic or *cyclo*hexanecarboxylic acids. The acids containing five-membered heterocyclic rings were more toxic than benzoic acid, the thiophene and furane rings being comparable. A β-carboxyl was more effective than α- in both napthoic and thiophenic acids. Among the five diphenyl and diphenoxyacetic acids, the most toxic was di-*p*-chlorophenylacetic acid, an oxidation product and metabolite of the insecticide DDT (White & Sweeney, 1945).

Table 2. *Structural specificity for aromatic acid toxicity*

The figures are the molar LD_{50} values × 10^3, followed by the confidence limits for 19/20 probability.

***para* Substituents in benzoic acid**		
Iodo	4·9	(4·2–5·8)
Chloro	8·7	(7·7–9·8)
Bromo	9·3	(8·3–11)
Fluoro	14	(13–16)
Methyl	22	(19–25)
Hydrogen	23	(19–27)
Methoxy	25	(23–27)
Amino	29	(26–32)
Nitro	30	(27–34)
Hydroxyl	51	(45–58)

Halogenated benzoic acids

	Chloro		Iodo	
2	24	(22–26)	—	
3	10	(9·2–11)	—	
4	8·7	(7·7–9·8)	4·9	(4·2–5·8)
2, 4	15	(13–17)	—	
2, 5	17	(15–20)	25	(21–30)
2, 6	61	(55–67)	—	
3, 4	5·3	(4·7–6·0)	3·7	(3·4–4·0)
3, 5	—		1·4	(1·3–1·5)
2, 3, 5	15	(13–17)	5·3	(4·5–6·1)
3, 4, 5	—		0·29	(0·27–0·30)
2, 3, 4, 5, 6	11	(10–12)	—	

Ring variation

Benzoic	23	(19–27)
*Cyclo*hexanecarboxylic	63	(53–75)
Nicotinic	46	(40–54)
1-Naphthoic	35	(30–40)
2-Naphthoic	12	(10–14)
2-Furoic	11	(9·1–13)
2-Thiophenecarboxylic	13	(11–15)
3-Thiophenecarboxylic	9·4	(8·1–11)

Side chain variation: R =

R.COOH	23	(19–27)
4-chloro	8·7	(7·7–9·8)
$R.CH_2.COOH$	26	(23–30)
4-chloro	9·7	(8·4–11)
$R.CH_2.CH_2.COOH$	37	(32–43)
R.CH=CH.COOH	29	(28–31)
$RO.CH_2.COOH$	69	(66–72)
4-chloro	46	(42–50)
$RO.CH_2.CH_2.COOH$	42	(37–48)
RCHO	25	(24–26)
RCH_2OH	5·5	(5·2–5·8)
$R.CO.NH.CH_2.COOH$	340	(300–390)
RSO_2H	440	(410–480)
RPO_3H_2 (3-chloro)	280	(260–300)

Miscellaneous aromatic acids

Diphenylacetic	47	(41–54)
Di(*p*-chlorophenyl)acetic	0·20	(0·19–0·22)
Di(*p*-methoxyphenyl)acetic	41	(33–52)
Di(*p*-chlorophenoxy)acetic	14	(10–21)
Di(*o*-chlorophenoxy)acetic	33	(27–39)
Indole-3-acetic	52	(49–55)
2-Furanacrylic	45	(39–52)

DISCUSSION

Studies of structural specificity of amino acids for the detoxication of aromatic acids have been limited previously by the lack of a suitable assay system. It is known that detoxication reactions involving glycine, ornithine and glutamine may occur in different animals (Williams, 1949). The results from this study with mosquito bioassay should be of particular interest, since they greatly expand the amino acids known to be effective in reducing the toxicity of aromatic acids. It is not known which ones act directly and which require a preliminary metabolic conversion into the effective counteracting agent. The structural specificity of this interaction was previously discussed, but several points seem worthy of further consideration. Asparagine and hydroxyproline, two of the poorer amino acids in counteraction, are not found in mosquitoes (Clark & Ball, 1952; Micks & Ellis, 1951, 1952), whereas the more efficient glutamine and proline are. Also, proline is metabolized in certain organisms to yield glutamic acid (which was shown to be very good in counteraction), whereas hydroxyproline is not (Umbreit, 1952). Decreases in efficiency with chain length are possibly due to penetration difficulties. Other still unexplained points of interest are: the poor relative efficiency of the thio amino acids compared with the hydroxy amino acids; the better efficiency of glutamic than of aspartic acid; the better efficiency of dicarboxylic acids than of their amides; and the better action of arginine than of citrulline.

The activities of benzoic acid and its derivatives have been studied with many biological systems, but in no case where adequate studies have been made for comparison is the relation of structure to toxicity the same as for the mosquito larvae. Structural specificity for the inhibition of the most sensitive enzyme reported, D-amino acid oxidase (Bartlett, 1948), varies greatly from the specificity for this insect bioassay. However, this does not rule out the possibility that the same physiological mechanism is involved in the toxicity, since the barrier of the insect cuticle may account for certain of the specificity differences. Many insecticides display their greatest toxicity in the non-ionized form (Brown, 1951), as was the case with benzoic acid in this study, due to the inability of ionized molecules to penetrate through the insect cuticle readily.

The interaction of different benzoic acid and amino acid levels illustrates a synergistic action of insecticides and other toxicants. Glycine, arginine and many other amino acids naturally present in insects were shown to reduce the toxicity of benzoic acid. At non-toxic concentrations, *p*-hydroxybenzoic acid increased the toxicity of benzoic acid. Both could presumably be involved in a glycine conjugation reaction. The enzymic conjugation forming *p*-aminohippuric acid may be inhibited by structurally related compounds including hydroxybenzoic acids (McKinney, 1951). It would appear that *p*-hydroxybenzoic acid might have effected its synergistic action by competition for the detoxication sites with benzoic acid, and in so doing reduced the amount of benzoic acid lost to detoxication and thereby increased its toxicity. This would be an example of synergistic action through competition for a common detoxication mechanism.

SUMMARY

1. The toxicity of benzoic acid to *Aedes aegypti* L. larvae was reduced in the presence of glycine. The efficiency of this counteraction decreased as the larvae matured and was greatest at pH 7·0 or below. The quantitative relationship of the decrease in benzoic acid toxicity in the presence of low glycine concentrations suggested an equimolar detoxication reaction. Benzoic acid was 15 times as toxic as hippuric acid to these larvae. Using [α-^{14}C]glycine it was shown that mosquito larvae can synthesize hippuric acid and probably excrete the conjugated product.

2. Thirty amino acids and closely related compounds were tested for their efficiency in reducing the toxicity of benzoic acid. Although glycine was the most efficient amino acid, in its absence many others were capable of reducing the toxicity of benzoic acid. The structural specificity for this counteraction is discussed.

3. The toxicity of fifty different aromatic acids or closely related compounds was determined. The relation of structure to the toxicity and mechanism of action of these acids is discussed.

4. An example is presented for a synergistic action through competition for a common detoxication mechanism.

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Canavanine: Detection and Occurrence in *Colutea arborescens*

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Canavanine, α-amino-δ-guanidinoxybutyric acid, is the only known example of a natural compound containing the guanidinoxy function,

$$—O.NH.C(:NH).NH_2.$$

Although canavanine occurs free to the extent of 2–3·5 % of dry weight in the seeds of the jack bean, *Canavalia ensiformis*, and the closely-related *C. lineata*, its discoverer, M. Kitagawa, was unable to obtain it from any other leguminous plant (Kitagawa & Tomiyama, 1929; Kitagawa, 1937). It has, however, been found in the seeds of *C. obtusifolia* (Damodaran & Narayanan, 1939).

During a study of the colour reactions of pentacyanoferrate derivatives and substituted guanidines, it was observed that guanidinoxy compounds, including canavanine, reacted only within the range pH 5–7·5, whereas the alkyl-substituted guanidines, including arginine, reacted only in solutions more alkaline than pH 8 (Fearon, 1946). This is in agreement with the observations of Kitagawa (1937) and Archibald (1946). As the result of an application of the pentacyanoferrate reactions to a survey of plant tissues, it has now been possible to show the presence of a guanidinoxy compound in some species of *Medicago* and *Ornithopus*, and canavanine has been isolated from the seeds of the bladder senna, *Colutea arborescens*.

EXPERIMENTAL

Reagents

A freshly prepared neutral aqueous solution of disodium pentacyanonitrosoferrate (sodium nitroprusside) does not react chromatically with a guanidoxine until the mixture has been exposed to daylight for about 5 min., when a colour, ranging from orange-red (*N*-methoxyguanidine) to magenta (canavanine) develops, and may persist for days. The pigment is stable to dilute acid, but is reversibly bleached by alkali. Alternatively, an aqueous solution of nitroprusside may be activated by irradiation before doing the test (Kitagawa & Yamada, 1932), though this seems to

offer no advantage over the direct method here described. In alkaline solution, photoactivated nitroprusside develops red colours with guanidine (Tiegs, 1924), urea (Ramsden, 1931) and other amidines, but these reactions are apt to be obscured by the chromatic versatility of the unchanged nitroprusside in presence of alkali.

Activation of the nitroprusside can be effected also by oxidizing agents in alkaline solution, including ferricyanide (Marston, 1925; Weber, 1928), persulphate (Pittarelli, 1928), Br_2 (Grote, 1931), I_2 (Fearon, 1937) and H_2O_2 (Archibald, 1946). The target of all these reagents is determined by the pH of the final mixture. In neutral or slightly acid solution, 'activated nitroprusside', however prepared, reacts characteristically with guanidinoxy compounds. The reagent chromogen in the mixture appears to be the ferric complex, pentacyanoaquoferrate (III), $[Fe(CN)_5OH_2]^{2-}$, formed by oxidation of pentacyanoaquoferrate (II), $[Fe(CN)_5OH_2]^{3-}$ a decomposition product of nitroprusside (Baudisch, 1948; van Sonsbeek, 1950; Fearon, 1946; Archibald, 1946). An objection to the use of 'activated nitroprusside' reagents is their instability and their liability to be contaminated by unchanged nitroprusside or by excess of activator. This can be overcome by employing a solution of trisodium pentacyanoammonioferrate, which can be prepared from sodium nitroprusside by Hofmann's method (Fearon, 1946; Herington, 1953) or obtained commercially.

Pentacyanoammonioferrate reagent (PCAF)

$Na_3[Fe(CN)_5NH_3]$ (1%, w/v) in Cu-free distilled water. The freshly prepared reagent is in the ferrous state and is of low chromogenic activity, as noted by Archibald. After exposure to light and air for about 1 hr., it begins to undergo spontaneous oxidation. The resulting mixture of the ferric and ferrous complex is relatively stable and retains its potency for at least a month, if stored in a brown glass bottle at low temperature.

Phosphate buffer (pH 7)

0·2 N-NaOH (29·54 ml.) and 0·2 M-KH_2PO_4 (50 ml.) are made up to 500 ml. with water and stored at low temperature.

Canavanine standard. Aqueous solution of canavanine base (1·0 mg./ml.) or the equivalent of canavanine sulphate. The salt can be prepared from jack bean meal or obtained commercially (Bios Laboratories Inc., 17 West 60th Street, New York 23, N.Y., U.S.A.; California Foundation for Biochemical Research, 3408 Fowler Street, Los Angeles 63, California, U.S.A.; L. Light and Co., Colnbrook, Bucks.). The standard solution is preserved by addition of toluene and storage at low temperature.

Test

About 5 ml. of the test solution, buffered to pH 7, are mixed with 5 drops of PCAF. The presence of canavanine is shown by the gradual development of a purple colour. If the reagent has been only recently prepared, addition of a drop of 1% (w/v) aqueous potassium persulphate will hasten colour formation. Apart from canavanine, the test is negative with the natural amino acids, including arginine and arginine peptides (salmine, clupeine), citrulline, cysteine, cystine and methionine. On making the mixture strongly alkaline by addition of 5 drops of 5 N-NaOH, the canavanine colour is discharged, but may be replaced by the development of a secondary colour if other amidines are present in the solution: orange-red (acetamidine, cyanamide), red (guanidine, urea) or red-purple (methylguanidine, glycocyamine, creatine, dicyanodiamide, galegine, arginine, streptomycin). A blue colour, developing both in neutral and in alkaline solutions, is given by some sulphur compounds containing S-S and S-O groupings (thiosulphate, thiocyanate, thiourea, formamidine disulphide, thiouracil, 4-methylthiouracil).

Sensitivity. At pH values below 4 and above 9, no colour is given by *N*-methoxyguanidine or by canavanine. The characteristic reaction begins about pH 5, and increases in rate of development and in final intensity as the pH rises to 7. At pH 7–8 the colour comes up rapidly and soon fades. At pH 6·5–7 the pigment has maximum stability, and may persist for at least a week at room temperature. In this pH range, the test will detect canavanine in concentrations as low as 0·008%.

Application to plant tissue

For each test, about 500 mg. of the fresh or dried material were triturated with 5–10 ml. of the pH 7 buffer and 5 to 10 drops of PCAF. The appearance of a red-to-purple colour within 10 min. was recorded as a positive test.

Guanidinoxy reaction negative. No colour reactions were obtained with the seeds, leaves, or fruit of the following species:

Amaryllidaceae: *Alstroemeria chilensis*; Balsaminaceae: *Impatiens noli-me-tangere*; Boraginaceae: *Borago officinalis, Lithospermum arvense, Myosotis arvensis*; Celastraceae: *Euonymus latifolius*; Chenopodiaceae: *Chenopodium bonus henricus*; Compositae: *Carthamus lanatus, Dahlia variabilis, Lapsana communis, Tragopogon porrifolius*; Cruciferae: *Biscutella leiocarpa, Brassica oleracea, Bunias orientalis, Crambe maritima*; Gramineae: *Avena sativa, A. sterilis, Hordeum distichon, Triticum vulgare*; Labiatae: *Phlomis alpina, Melittis melissophyllum, Stachys germanica*; Leguminosae: *Desmodium tiliifolium*; Liliaceae: *Camassia esculenta, Scilla hispanica*; Onagraceae: *Eucharidium concinnum*; Papaveraceae: *Papaver rhoeas*; Primulaceae: *Cyclamen hederaefolium*; Ranunculaceae: *Clematis vitalba*; Resedaceae: *Reseda luteola*; Rosaceae: *Cotoneaster frigida, Geum rivale*; Saxifragaceae: *Tellima grandiflora*; Scrophulariaceae: *Scrophularia orientalis*; Solanaceae: *Browallia* sp., *Solanum dulcamara*; Umbelliferae: *Eryngium oliverarium, Perula communis*; Valerianaceae: *Valeriana* sp.

Guanidinoxy reaction positive:

Leguminosae: *Colutea arborescens, Medicago arborea, M. echinus, Ornithopus perpusillus.*

Colutea seed was found to give an intense colour with PCAF, and as the seeds could be obtained in sufficient quantity, they were submitted to the extraction procedure that had proved satisfactory in the preparation of canavanine from jack bean.

Isolation and characterization of canavanine from Colutea arborescens

The meal from 113 g. of finely ground seed was suspended in 400 ml. of distilled water and acidified to Congo red paper by dropwise addition of conc. H_2SO_4. After remaining overnight at room temp., the pasty suspension was mixed with 1 vol. of water, stirred and filtered, to remove substances that interfered with the subsequent fractionation. The

residue was then extracted thoroughly with an equal volume of water and the filtrate, which gave a strongly positive PCAF test, was concentrated to about 200 ml. on a water bath. After filtration, the concentrate was mixed with 5 vol. ethanol and kept overnight at 5°. After decantation of the ethanol, the viscid residue adhering to the sides of the flask was rinsed out with a minimum amount (50 ml.) of cold water, and treated with an equal volume of cold 20% (w/v) aqueous flavianic acid. After keeping overnight at 5°, the crystalline flavianate which had formed was removed by filtration and recrystallized 4 times from hot water. It was then dissolved in 50 ml. hot water and treated drop wise with hot saturated aqueous $Ba(OH)_2$, until no further separation of Ba salts could be observed and the mixture was alkaline (pH 9·5). After removal of the barium flavianate by filtration, the filtrate was acidified carefully to Congo red by addition of H_2SO_4, to precipitate excess Ba^{2+}, and boiled with activated charcoal (British Drug Houses Ltd.), to remove traces of flavianic acid. The resulting colourless filtrate was concentrated to about 45 ml. on a water bath and, when cold, treated with 4 vol. of ethanol. A clear syrup separated on standing. After decantation of the supernatant layer, the residue was suspended in warm ethanol, to which water was added drop by drop, until, aided by warming and stirring, complete solution took place. The solution was allowed to cool slowly, and then transferred to a refrigerator. After keeping at 5° for 48 hr., a crop of colourless prismatic crystals had separated, and was transferred to a desiccator. Yield, 1·2 g. The crystals began to shrivel at 165° and melted with decomposition at 172°. Canavanine sulphate from jack bean (Kitagawa, 1937) had m.p. 172° (decomp.); (E.A.B.) 172° (decomp.). No depression of m.p. was observed when the *Colutea* compound was mixed with a specimen of canavanine sulphate from jack bean. (Found: C, 20·5; H, 5·4; N, 19·7. Calc. for $C_5H_{12}O_3N_4$, H_2SO_4, H_2O: C, 20·5; H, 5·5; N, 19·2%.) Carboxyl-N (Van Slyke, MacFadyen & Hamilton, 1941), 4·56. Calc. for $C_5H_{12}O_3N_4$, H_2SO_4, H_2O: 4·79%. The compound was very soluble in water, giving a solution acid to Congo red. $[\alpha]_D^{18\cdot5}$, +18·62° in water (*c*, $C_5H_{12}O_3N_4$, H_2SO_4, $H_2O = 7{\cdot}76\%$ (w/v)). Calc. for $C_5H_{12}O_3N_4$, H_2SO_4: +19·84°. Canavanine sulphate from jack bean (Kitagawa) $[\alpha]_D^{17}$, +19·41°).

Filter-paper chromatography (upward running on Whatman no. 4 paper at 18±1°) gave a single spot in phenol–water (R_F, 0·26) or in *n*-butanol–acetic acid–water (4:1:5, by vol.) (R_F, 0·13). The chromatograms were coloured by spraying with 0·4% ninhydrin in water-saturated *n*-butanol or with PCAF mixed with an equal vol. of pH 7 buffer or with 1% (w/v) aqueous sodium nitroprusside followed by exposure to daylight. Canavanine sulphate from jack bean behaved in the same way in all respects.

From these results it is concluded that the guanidinoxy compound present in *Colutea arborescens* is canavanine.

Colorimetric estimation of canavanine

To establish the working conditions, a curve showing the relationship between the optical density of the colour with PCAF and the concentration of canavanine was prepared by setting up a series of colorimeter tubes containing, respectively, 0·2, 0·4, 0·6, 0·8, 1·0, 1·4 and 2·0 ml. of canavanine standard. Each tube was treated with 0·5 ml. of PCAF and the volume made up to 10 ml. with pH 7 buffer. After mixing the contents, the tubes were exposed to diffused daylight for 40 min. and then compared in an EEL photoelectric colorimeter with a blue-green '303' filter (Evans Electroselenium Ltd., Harlow, Essex). A solution containing 9·5 ml. of buffer and 0·5 ml. of reagent was used as the 'blank' control. The results are shown in Fig. 1.

Unlike the original nitroprusside reaction, the PCAF reaction is not highly photosensitive. With an active solution of the reagent, colour development can proceed in complete darkness and a difference of 10 min. either way during the period of exposure to daylight was found to have no appreciable effect on the colorimeter readings. The speed of colour development, however, does depend on the extent to which the reagent has undergone spontaneous oxidation during storage.

Procedure. To 1 ml. of the neutral solution for test, containing 0·1–1·0 mg. of canavanine, is added 0·5 ml. of PCAF; the solution is made up to 10 ml. with pH 7 buffer, shaking the mixture during the dilution. A working standard is prepared at the same time, using 1 ml. of the canavanine standard. After 40 min. exposure to daylight, the solutions are compared by means of a visual or photoelectric colorimeter with a blue or blue-green filter.

Results. Canavanine (g. free base/100 g. dry weight): bladder senna seed, 4·7; jack bean seed, 3·3; soya bean seed, 0·0.

Interference effects. (1) *Reducing agents.* The colours obtained with pure canavanine and PCAF or light-activated nitroprusside are relatively stable; the colours given by aqueous extracts of jack bean meal and PCAF tend to fade in a few hours, irrespective of whether the mixtures are kept in the light or the dark. The colour returns on addition of more of the reagent or an oxidizer, such as H_2O_2 or persulphate. The fading effect is attributed to the presence

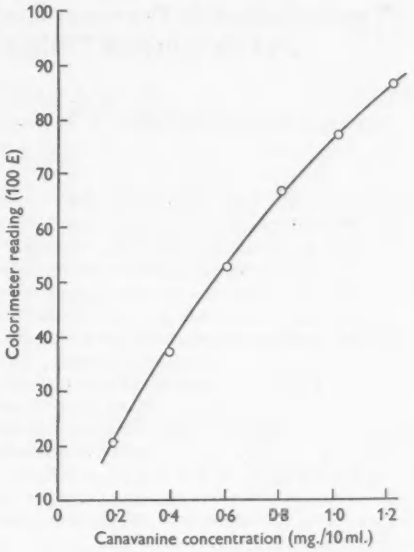

Fig. 1. Relation between optical density of the pentacyanoammonioferrate complex and the canavanine concentration.

of agents capable of reducing the ferric component in the reagent. Ascorbic acid, hydroxymethylglyoxal (reductone) and cysteine can bring about fading of the canavanine colour, and if present in sufficient quantity can inhibit completely the colour test. These, and possibly other reducing factors, must be considered in testing biological material for guanidinoxy compounds; their effects can be overcome by pretreatment with aqueous iodine.

(2) *Competitors.* No guanidinoxy reaction could be observed with any animal secretion or tissue examined (milk, blood serum, cerebrospinal fluid, urine, liver, muscle, kidney), and low recovery values were obtained for canavanine added to these materials. Pretreatment with aqueous iodine had little if any effect on the low recovery, which suggested that it was not caused by destruction of the canavanine by arginase or inactivation of the reagent by reducing agents. Individual tests in presence of the chief urinary solutes showed that creatinine strongly inhibits the PCAF reactions with guanidinoxy compounds. This inhibition can be overcome by addition of an excess of the reagent, which indicates that the creatinine inhibition effect may arise from competition with the canavanine.

SUMMARY

1. Methods are described for the detection and estimation of guanidinoxy compounds, including canavanine, in biological material.

2. Canavanine has been isolated from the seeds of the bladder senna, *Colutea arborescens*.

3. A guanidinoxy compound, similar in its colour reactions to canavanine, has been detected in the seeds of *Medicago arborea*, *M. echinus*, and *Ornithopus perpusillus*.

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The Influence of Previous Diet on the Fasting Blood-Sugar Level and on Glucose Utilization in the Rat and Hamster

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Observations have been made from time to time on the effect of previous diet upon carbohydrate metabolism in animals. Samuels, Gilmore & Reinecke (1948) found that, after short periods of fasting, rats on a high-fat diet had lower blood-sugar levels than those on a high-carbohydrate diet. A reduced tolerance in man to orally or intravenously administered glucose (Sweeney, 1927; Himsworth, 1933) and depressed glucose utilization by isolated rat diaphragm (Lundbaek & Stevenson, 1948; Gilmore & Samuels, 1949; Hansen, Rutter & Samuels, 1951) has also been observed as a result of feeding a diet high in fat and low in carbohydrate. In these respects, fat-fed non-ruminant animals show a striking resemblance to the adult ruminant (Reid, 1950*a*, *b*; 1952).

The purpose of the present work was to confirm these findings in rats and hamsters with a view to reproducing the conditions obtaining in the adult ruminant in non-ruminant animals by suitable modification of the diet. The effect of feeding various diets upon the fasting blood-sugar level, upon glucose tolerance, and upon glucose utilization and glycogen deposition in isolated diaphragm has therefore been examined. Studies on the hexokinase activity of various tissues were included in the hope that they would throw light upon the mechanisms involved.

While the work reported here was in progress, Long (1953) published his results on the hexokinase activity of tissues from rats fed from weaning on a variety of diets. Using homogenates of intestinal mucosa, highest activity was found in animals fed a high-carbohydrate, fat-free diet and lowest in those fed a high-fat, carbohydrate-free ration. As recorded below, similar results have been obtained using homogenates of rat and hamster kidney.

EXPERIMENTAL

Animals and diets

Hooded rats and hamsters were used. They were introduced to the synthetic diets (Table 1) immediately after weaning (at 22 days old). The mean weights of the rats after 13 weeks on the high-carbohydrate, high-fat and high-protein rations were 164, 133 and 136 g., respectively.

Experiments with isolated diaphragm

Animals which had been maintained on the appropriate diet for 3 months were fasted for 48 hr. (this rather lengthy period of starvation being found necessary to obtain approximately the same initial glycogen content in each diaphragm) and killed by decapitation and exsanguination. The diaphragm was immediately removed and placed in ice-cold Krebs phosphate–Ringer solution. Warburg flasks to receive the hemidiaphragm contained, in the main compartment, 2·5 ml. phosphate–Ringer with glucose (80 mg./100 ml.), and 0·2 ml. 10% (w/v) KOH in the centre well. The diaphragm was divided along the mid-line, and the two portions placed in separate Warburg vessels. The flasks and attached manometers were gassed for 15 min. with O_2 and then placed in the water bath at 38° for 10 min. to equilibrate. At the end of this period one-half of each diaphragm was removed for determination of the initial glycogen content. The other half of the diaphragm was incubated for a further 2 hr., the flasks being shaken at approximately 100 oscillations/min. The hemidiaphragm was then removed for glycogen determination. The initial and final glucose concentrations of the medium were determined in 0·5 ml. of fluid.

Determination of hexokinase activity

The kidneys and brain from the animals used for the isolated diaphragm experiments were removed as rapidly as possible and immersed in ice-cold water. The tissues were then homogenized with phosphate–fluoride buffer in a glass-Perspex homogenizer of the Potter & Elvehjem (1936) type; the technique employed was essentially that described by Long (1952). The incubation fluid contained the following components (final concentrations in brackets): glucose (0·0012 M), potassium adenosine triphosphate (ATP) (0·005 M), KF (0·05 M), potassium phosphate buffer, pH 7·8 (0·04 M), $MgCl_2$ (0·005 M) and KCl (0·042 M). The amount of glucose disappearing in 5 min. (brain) or 10 min. (kidney) at 30° in air was determined by the Harding-Nelson method (King & Garner, 1947). Hexokinase activity was estimated by the difference in glucose utilization in corresponding preparations with and without ATP.

Determination of glucose and glycogen

Blood glucose was determined by the titrimetric method described by King (1951) on a protein-free filtrate prepared according to Somogyi (1945). Blood for glucose determination was obtained by heart puncture under Nembutal (sodium pentobarbitone) anaesthesia, precipitation of proteins being carried out within 1–2 min. of obtaining the blood sample. Glycogen determinations were made by the method of Good, Kramer & Somogyi (1933).

RESULTS

Effect of previous diet on fasting blood-sugar levels

In both rats and hamsters, the blood-sugar level of fat-fed animals is lower after 17 hr. fasting than that of corresponding controls receiving the diet containing 56% carbohydrate (Table 2*A*). The differences recorded in rats are statistically significant at both 7 and 10 weeks ($P < 0·001$). As is also shown in this table, the blood sugar of the fat-fed animals can be restored to the level of that of the controls by reversing the diets.

Blood samples were also taken from three groups of thirteen rats before killing for removal of diaphragm and kidneys. These animals had been maintained on high-carbohydrate, high-fat and high-protein diets (nos. 3–5, Table 1) for 3 months and the mean figures for blood glucose were 85, 89 and 99 mg./100 ml., respectively. The results previously obtained are apparently completely reversed. However, these rats had been starved for 48 hr. as compared with 17 hr. for the first set. Since Samuels *et al.* (1948) had found that rats receiving a high-fat diet exhibited lower blood-sugar levels than those on a high-carbohydrate diet only for the first 6 hr. of fasting, the position being reversed after 12–16 hr., it seemed likely that the lengthier period of fasting was responsible for the discrepancy. This was confirmed by taking blood samples after 17, 24 and 41 hr. fasting (Table 2*B*).

Table 1. *Composition of diets*

One part of monotype punchings was added to 19 parts of diet. The salt mixture was prepared according to Osborne & Mendel (1913).

	Diet (g./100 g.)				
	No. 1 High carbohydrate	No. 2 High fat	No. 3 High carbohydrate	No. 4 High fat	No. 5 High protein
Lard	5·7	32	—	—	—
Arachis oil	4·3	24	—	—	—
Hydrogenated palm oil	—	—	9	56	9
Starch	56	10	57	10	10
Casein	23	23	23	23	70
Marmite	5	5	5	5	5
Cod-liver oil	1	1	1	1	1
Salt mixture	4	4	4	4	4
NaCl	1	1	1	1	1

Table 2. *Effect of feeding high-carbohydrate and high-fat diets from weaning on fasting blood-sugar levels of rats and hamsters*

Figures given are mean ±S.D.

A. Effect of diet on changes in 17 hr. fasting blood-sugar level with age

Diet	No. of animals	Blood glucose (mg./100 ml.) at (weeks) 4	7	10
Rats				
No. 1 (high carbohydrate)	11	70± 8·4	106±3·3	100±5·5
No. 2 (high fat)	11	70±10·9	85±6·9	89±6·8
Diets interchanged at 7 weeks				
No. 1 (high carbohydrate)	6	66±5·8	105±13·6	88±11·6
No. 2 (high fat)	6	58±5·2	90±11·8	105± 9·3
Hamsters				
No. 3 (high carbohydrate)	5	87±2·4	72±17·7	67±15·1
No. 4 (high fat)	6	85±1·8	60±14·2	60±25·3

B. Effect of diet on changes in blood-sugar level of 13 week-old rats with varying periods of fasting

Diet	No. of animals	Blood glucose (mg./100 ml.) after fasting (hr.) 17	24	41
No. 3 (high carbonhydrate)	5	84± 9·1	68± 6·3	60±14·4
No. 4 (high fat)	6	74±11·4	77±12·8	67± 7·0

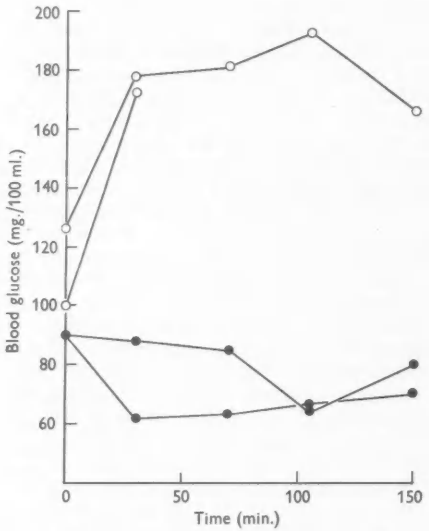

Fig. 1. Effect of previous diet on tolerance to intravenous glucose (0·3 g./kg.) in rats; ●, high-carbohydrate diet; ○, high-fat diet.

Effect of previous diet on glucose tolerance in rats

Four 4-month-old rats (two on high-carbohydrate diet no. 1, two on high-fat diet no. 2) which had been fasted for 17 hr. were used. Under Nembutal anaesthesia a blood sample was taken by heart puncture and then, without removing the needle, 25% (w/v) glucose solution was injected at the rate of 0·3 g./kg. body weight. Further blood samples were taken from the heart over a period of 2·5 hr. The results are recorded in Fig. 1. This procedure was invariably fatal, one animal dying during the course of the test and the remaining three a few hours after the test had been completed, presumably due to repeated damage to the heart.

Effect of previous diet on glucose utilization and glycogen synthesis in isolated rat diaphragm

In a preliminary experiment using three rats from each of the groups on diets nos. 1 and 2 (high-carbohydrate and high-fat, respectively) it was confirmed that fat feeding depresses both glucose utilization and glycogen synthesis (Table 3). In order to determine whether the effect was due to the high fat or low carbohydrate content, a further experiment was carried out including an additional group of rats on a diet containing 70% protein (diet no. 5, Table 1). The results are summarized in Table 3.

Effect of previous diet on the hexokinase activity of brain and kidney homogenates

Two groups of rats and one group of hamsters, all of which had been maintained on the appropriate diets for 3 months were used. The animals were fasted for 48 hr. and duplicate (later triplicate) estimations on kidney and, in some cases, brain activity of one animal on each diet were carried out simultaneously. Table 4 lists the mean results for all groups.

Table 3. *Effect of previous diet on glucose utilization and glycogen synthesis by isolated rat diaphragm*

Figures given are mean ±S.D. Glucose and glycogen are expressed as mg. glucose/100 mg. wet weight of tissue/hr.

Diet	No. of experiments	Glucose used	Glycogen synthesized
No. 1 (high carbohydrate)	3	0·220±0·019	0·044±0·014
No. 2 (high fat)	3	0·180±0·047	0·006±0·016
No. 3 (high carbohydrate)	7	0·212±0·077	0·031±0·024
No. 4 (high fat)	7	0·210±0·156	0·018±0·019
No. 5 (high protein)	7	0·170±0·042	0·012±0·020

Table 4. *Effect of previous diet on hexokinase activity of brain and kidney homogenates*

Figures given are mean ±S.D. Hexokinase activity is expressed as mg. glucose disappearing/100 mg. wet wt./5 min. (brain) or 10 min. (kidney).

	No. of animals in each group	Diet: No. 1 (high carbohydrate)	Diet: No. 2 (high fat)
Rat brain	8	0·405±0·0672	0·414±0·0882
Rat kidney	8	0·241±0·0365	0·225±0·0142

	No. of animals in each group	No. 3 (high carbohydrate)	No. 4 (high fat)	No. 5 (high protein)
Rat kidney	11	0·198±0·0460	0·142±0·0364	0·250±0·0722
Hamster kidney	2	0·198±0·0178	0·128±0·0282	—

DISCUSSION

The effect of varying the period of fasting on the blood-sugar level of rats on high-carbohydrate and high-fat diets is similar to, but not identical with, that described by Samuels *et al.* (1948). From an inspection of Table 2*B* it is apparent that similar blood-sugar values would be reached in the two groups of rats after approximately 20 hr. fasting, as opposed to 6–12 hr. as found by Samuels *et al.* As mentioned above, it was found necessary to starve the rats used in the present work for 48 hr. in order to reduce the initial glycogen content of the diaphragm to similar levels in all animals. It was also found that up to 72 hr. fasting was required to deplete the liver glycogen of similar rats on a stock diet. This is considerably longer than the 24 hr. usually considered necessary, and may reflect some peculiarity of the particular strain used. As it is during the period of glycogen depletion that the blood sugar falls, the discrepancy between the results reported here and those of Samuels *et al.* may be due simply to strain differences.

The fall in blood sugar with age seen in hamsters is of considerable interest as it is believed that this phenomenon is otherwise confined to ruminants and related species. The plasma glucose concentration of the fat-fed animals at 10 weeks appears to be of the same order as that recorded for the adult ruminant (Somogyi, 1933).

All workers agree that glucose utilization is depressed by high-fat, low-carbohydrate diets and this reduced utilization is clearly demonstrated by the intravenous glucose-tolerance tests (Fig. 1) and less clearly by the isolated diaphragm experiments. It may be argued that the reduced tolerance to glucose in the fat-fed rats reflects a terminal rise in blood sugar, but no similar rise occurred in the controls, which also died a few hours after the test had been completed.

Glycogen synthesis by rat diaphragm has been studied by several workers but the results obtained have been equivocal. According to Lundbaek & Stevenson (1948) the previous diet has no effect on the amount of glycogen synthesized, which suggests that a high-fat diet exerts a sparing action on glucose oxidation. However, Hansen *et al.* (1951) report that less glycogen is formed on a high-fat diet, although again their results indicate a sparing action. In the present work glycogen synthesis has been found to be profoundly depressed by both high-fat and high-protein diets, indicating that it is carbohydrate deprivation which is the operative factor. Some of these discrepancies are capable of an explanation. Hansen *et al.* believe that the initial glycogen content of the diaphragm determines to some extent whether glycogen synthesis or breakdown shall occur. In the light of observations made during the course of the work presented here it is considered that the glucose level of the blood may also be a determining factor.

Depressed glucose utilization suggests depressed hexokinase activity. The negative findings in the first series of rats used (Table 4) are difficult to explain in view of the positive results obtained with kidney preparations from the second series of rats and from hamsters. The reduction in activity has since been confirmed in a different strain of rats

during the course of further work to be reported at a later date. It is certain that there are differences in the effect of diet on different tissues as Long (1953) has found that, although the hexokinase activity of intestinal mucosa is depressed by a low-carbohydrate diet, that of intestinal muscle is unaffected.

In so far as attempts to reproduce the anomalies of carbohydrate metabolism observed in the adult ruminant are concerned, these have been successful and it may be concluded from the similarities between the adult ruminant and the fat-fed non-ruminant that many of the apparent abnormalities in the former are due to the peculiar digestive processes of the ruminant rather than to specific hormonal or enzymic differences.

SUMMARY

1. After 17 hr. fasting, blood-sugar levels from rats and hamsters fed on a high-fat diet from weaning are consistently lower than those from similar animals on a high-carbohydrate diet.

2. The reduced tolerance to intravenously injected glucose induced by a high-fat diet has been confirmed using rats.

3. Restriction of the carbohydrate intake by feeding excess fat or protein depresses both glucose uptake and glycogen synthesis by isolated rat diaphragm.

4. The hexokinase activity of kidney homogenates from rats and hamsters is depressed by high-fat diets.

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Evidence for the Occurrence of γ-Methylene-α-oxoglutaric Acid in Groundnut Plants (*Arachis hypogaea*)

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The isolation of two new amino acids, γ-methyleneglutamic acid and γ-methyleneglutamine, from groundnut plants (*Arachis hypogaea*) was described by Done & Fowden (1951, 1952). More recently these two compounds have been isolated from tulips (*Tulipa gesneriana*) by Zacharius, Pollard & Steward (1954), and their presence has been indicated in hops (*Humulus lupulus*) by Harris & Tatchell (1953). Although these compounds have a limited distribution within the plant kingdom, there are indications that they are of considerable importance in the metabolism of those species in which they occur. γ-Methyleneglutamic acid can, under the influence of the transaminase systems present in groundnut plants, transfer its α-amino group to α-oxoglutaric, oxalacetic or pyruvic acids (Fowden & Done, 1953*a*), whilst γ-methyleneglutamine appears to be important in the transport of nitrogen within this species (Fowden, 1954).

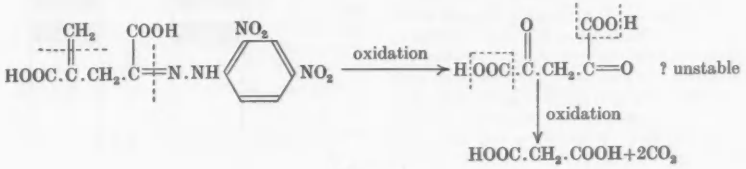
α-Oxo acids have been the subject of several detailed investigations, and α-oxoglutaric, oxalacetic, pyruvic and glyoxylic acids have, by characterization as their 2:4-dinitrophenylhydrazine (DNPH) derivatives, been found to be widely

distributed in plant and animal tissues. Recently Steward and his co-workers, using a new method for the identification of DNPH derivatives, have obtained preliminary evidence for the presence of additional keto acids in plants, and detected the keto-acid analogues of valine and γ-methyleneglutamic or γ-methylglutamic acids (Towers, Thompson & Steward, 1954). Towers & Steward (1954) claim to have isolated the latter keto acid, as its DNPH derivative, from tulips. After completion of the transamination studies using groundnut extracts referred to above, we considered γ-methylene-α-oxoglutaric acid to be a possible constituent of groundnut plants, and commenced an investigation of the α-keto acids present in groundnut seedlings. We now report the isolation of this unsaturated keto acid as its DNPH derivative, together with more rigorous proof of its chemical identity than has so far been published. A brief description of some of the present experiments has appeared earlier (Fowden & Webb, 1954).

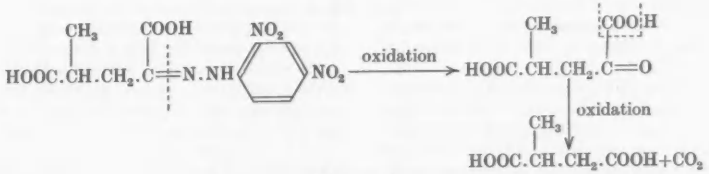
Preliminary experiments were performed to reveal the number and probable nature of the keto acids present in six-day-old seedlings. Aqueous deproteinized extracts of the seedlings were treated to form the DNPH derivatives of the keto acids, which were then separated on paper chromatograms. One strong spot, moving just in advance of that of the derivative of α-oxoglutaric acid, was not attributable to any known keto acid, but did occupy the same position on chromatograms as the derivative of the keto acid (presumably γ-methylene-α-oxoglutaric acid) arising from γ-methyleneglutamic acid by biological transamination.

Having established the probable nature of the new keto acid, larger quantities of seedlings were extracted, and about 12 mg. of the pure DNPH derivative of the acid finally obtained, after it had been separated from the derivatives of other keto acids by cellulose-powder column and paper-chromatographic procedures. Since the keto acid has not been chemically synthesized, a biologically synthesized reference substance was prepared by transamination from γ-methyleneglutamic acid and α-oxoglutaric acid; the structure of this amino acid has now been confirmed by chemical synthesis (Wailes, Whiting & Fowden, 1954). The natural and synthetic DNPH derivatives so obtained behaved identically when subjected to the following treatments: (*a*) paper chromatography, (*b*) melting-point determinations, (*c*) hydrogenation in the presence of Adam's catalyst, (*d*) visible spectra determinations, (*e*) infrared spectra determinations on Nujol mulls, (*f*) X-ray spectra determinations of their ammonium salts and (*g*) oxidation by hydrogen peroxide.

Hydrogenation, in yielding an amino acid identical with γ-methylglutamic acid, alone established that the new keto acid was probably γ-methylene- (or γ-methyl-) α-oxoglutaric acid. To ensure that reduction of the —C=C— bond had not accompanied transamination during the biological synthesis of γ-methylene-α-oxoglutaric acid from γ-methyleneglutamic acid (so yielding γ-methyl-α-oxoglutaric acid), the DNPH derivatives of the natural and synthetic acids were oxidized with hydrogen peroxide, and the products investigated by organic acid-paper chromatography. Malonic acid was the only oxidation product that could be so identified, and would be expected according to scheme A. The derivative of γ-methyl-α-oxoglutaric acid would be expected to yield methylsuccinic acid by similar treatment (see scheme B). A small quantity of this latter derivative, prepared by transamination from synthetic γ-methylglutamic acid, did in fact yield methylsuccinic acid as the main oxidation product.



Scheme A.



Scheme B.

EXPERIMENTAL

Plant material. Groundnut seeds were grown in moist sawdust at 25–30° for 6 days. Each seedling weighed about 2 g. fresh weight upon harvesting, and had developed a branched root system and the first two immature leaves.

Preparation of the DNPH derivatives of the keto acids. The seedlings, washed free from adhering sawdust, were macerated with an equal weight of distilled water in an 'Atomix' blender. The mush was strained through muslin, and the pH of the filtrate adjusted to approximately 4·2 with H_2SO_4. The proteins were coagulated by warming to 50°, and removed by centrifuging. The keto acids were converted into their DNPH derivatives by treatment of 10 vol. of the supernatant with 1 vol. of a saturated solution of 2:4-dinitrophenylhydrazine in 2N-HCl for 20 min. at 40°. The derivatives were extracted into ether by four successive extractions (1 vol. ether to 4 vol. supernatant), and the combined ether extracts reduced to one-fifth of their original bulk. The concentrated ether extract was then shaken with successive small volumes of 10% (w/v) Na_2CO_3 soln. until no further appreciable amounts of acidic DNPH derivatives entered the Na_2CO_3 layers. Unchanged 2:4-dinitrophenylhydrazine remained in the ether layer at this stage. The combined Na_2CO_3 layers, maintained at 0°, were made slightly acid by the addition of conc. HCl and the DNPH derivatives of the keto acids again completely transferred to ether. These extracts were combined and evaporated to dryness, yielding a residue consisting mainly of the DNPH derivatives of the keto acids, together with smaller amounts of dark-coloured resinous substances. This extraction procedure was modified from those published by El Hawary & Thompson (1953) and Cavallini & Frontali (1954).

Preliminary identification experiments

In these experiments 100 g. fresh weight of seedlings were treated as above to obtain the mixed DNPH derivatives of their oxo-acid constituents. The residue was dissolved in the minimum volume of 0·5N-NH_3 soln., and a measured fraction applied as a short band to a sheet of Whatman no. 3 filter paper. A similar band was applied adjacent to the first, and contained the derivatives of α-oxoglutaric, glyoxylic and pyruvic acids prepared from 40 μg. of each of the pure acids by a procedure similar to that above. The chromatogram was then developed for 20 hr. using *n*-butanol saturated with 6% (w/v) NH_3 soln. as the mobile phase (Fincham, 1953). The appearance of the chromatogram is reproduced in Fig. 1. The derivatives of pyruvic and glyoxylic acids each gave two spots on the developed chromatogram. The seedlings contained oxo acids co-chromatographing with α-oxoglutaric, pyruvic and glyoxylic acids, and four additional acids giving DNPH derivatives. One of these formed a strong band moving slightly in advance (R_F 0·14) of the α-oxoglutaric acid derivative, and was the subject of the further investigations to be described. The three other unknown derivatives formed fainter bands and moved at lower R_F values than α-oxoglutaric acid. Their nature has not been further investigated.

A small amount of the DNPH derivative of γ-methylene-α-oxoglutaric acid, admixed with a larger amount of the α-oxoglutaric acid derivative, was prepared by the procedure outlined above from the mixture of the two keto acids resulting from transamination between 2 mg. γ-methyleneglutamic acid (natural) and 2 mg. of α-oxoglutaric acid in the presence of a dialysed groundnut extract (reaction conditions as Fowden & Done, 1953*a*). The derivative of γ-methylene-α-oxoglutaric acid also flowed slightly in advance of that of α-oxoglutaric acid, and was inseparable from the unknown (R_F 0·14) after the chromatogram had been developed for 3 days in the *n*-butanol–NH_3 solvent system.

Larger-scale preparations

Isolation of the unknown DNPH derivative from seedlings. Groundnut seedlings (2 kg., fresh wt.) were treated by the above extraction procedure and yielded about 1 g. of residue containing the mixed DNPH derivatives. A preliminary purification of the unknown derivative was accomplished on a cellulose-powder column. Dry Whatman cellulose powder (200 g.), previously extracted with N-HCl followed by N-NaOH, was mixed with *n*-butanol saturated with 6% (w/v) NH_3 soln. and poured as a slurry into a column of 50 mm. diameter. Frequent tamping ensured tight packing, and gave a column of 290 mm. length. The DNPH derivatives were dissolved in 20 ml. of the same solvent, and applied slowly to the column. The column was developed with more solvent, slight suction being used to obtain a percolation rate of about 50 ml./hr. After 5 hr. the development was stopped, the unknown material having formed a separate, and fairly well-defined band about 8 cm. down the column. The DNPH derivative of α-oxoglutaric acid had also formed a fairly discrete band, but the derivatives of glyoxylic and pyruvic acids, which moved down the column more rapidly, remained incompletely resolved. Excess solvent was allowed to drain from the column, and

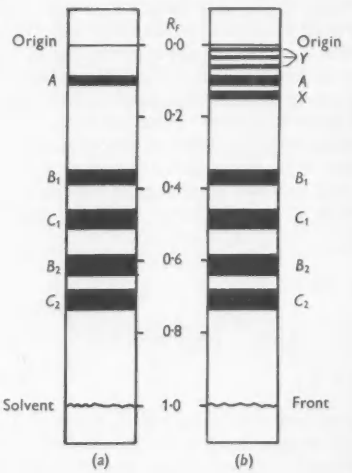


Fig. 1. Representation of a chromatogram on Whatman no. 3 filter paper developed in the *n*-butanol–NH_3 solvent system containing the DPNH derivatives prepared from (*a*) a standard mixture (containing 40 μg. each acid) of pure α-oxoglutaric acid (*A*), glyoxylic acid (B_1 and B_2) and pyruvic acid (C_1 and C_2), and (*b*) the keto acids occurring in extracts of six-day-old groundnut seedlings. *X* is the unknown keto-acid derivative investigated in this paper; *Y* represents three further unknown compounds which have not been investigated.

then the cellulose packing was carefully extruded to obtain the portion containing the unknown derivative. The remaining solvent was removed from this by drying *in vacuo* at laboratory temperature.

The derivative was extracted from the dried cellulose powder using four lots of 25 ml. of 0·5 N-NH_3 soln. The combined extracts were made slightly acid by addition of conc. HCl, and the derivative was then quantitatively transferred to ether. A light-yellow residue remained on removal of the ether. Paper chromatograms revealed that it consisted of the unknown, contaminated by small amounts of the derivative of α-oxoglutaric acid. The contaminant was removed by applying the residue in a 25 cm. band near to the top edge of a sheet of Whatman no. 3 filter paper and developing the chromatogram for 40 hr. in the *n*-butanol–NH_3 solvent system. Complete resolution of the two substances occurred, and the unknown derivative was eluted from the stronger band by 0·5 N-NH_3 soln. After acidifying the extract with conc. HCl, the derivative was again transferred to ether, and obtained as a yellow residue upon evaporation. This residue was re-extracted with dry ether (any NH_4Cl carried over into the former wet ether layers would remain undissolved), and the extract again evaporated to yield 12 mg. of the unknown.

Preparation of the DNPH derivative of γ-methylene-α-oxoglutaric acid by transamination. An extract of four-day-old groundnut seedlings was prepared by grinding them with washed sand and a small amount of 0·025 M potassium phosphate buffer, pH 7·4. The centrifuged extract was dialysed for 20 hr. against a stream of 0·05 M potassium phosphate buffer, pH 7·4 at 2°. No amino or keto acids could be detected in this dialysed extract, which was used as a source of transaminases. A 10 ml. portion of the extract was added to a mixture of 25 mg. of α-oxoglutaric acid and 25 mg. of γ-methyleneglutamic acid (natural), previously adjusted to pH 7·4 with NaOH and made up to 10 ml. with water. This reaction mixture was incubated at 37° for 4 hr.; at the end of this time determinations on measured fractions showed that glutamic and γ-methyleneglutamic acids were the only amino acids, and α-oxoglutaric and γ-methylene-α-oxoglutaric acids the only keto acids present in the mixture. The keto acids were converted into their DNPH derivatives and isolated as previously; 40 mg. of mixed derivatives resulted, about 70% consisted of the α-oxoglutaric acid derivative. The two derivatives were first separated on a 40 × 220 mm. cellulose column, followed by final purification of the γ-methylene-α-oxoglutaric acid derivative on paper chromatograms as described above for the unknown. The yield of the pure derivative was 10 mg.

Identity of the unknown derivative

The unknown derivative was shown to be identical with the biologically synthesized γ-methylene-α-oxoglutaric acid derivative by the following criteria.

Chromatographic behaviour. The two derivatives were inseparable on paper chromatograms developed in the *n*-butanol–NH_3 solvent for 5 days, during which time they had moved about 35 cm. down the paper.

Melting-point determinations. The unknown had m.p. 228° (decomp.), whilst the DNPH derivative of γ-methylene-α-oxoglutaric acid had m.p. 224° (decomp.). The mixed m.p. was 226° (decomp.). All m.p.'s were corrected.

Hydrogenation. Towers *et al.* (1954) showed that when DNPH derivatives of keto acids were hydrogenated in the presence of a suitable catalyst, the analogous amino acids were produced, and could be identified by paper-chromatographic methods. Solutions of about 1 mg. of the unknown and of the synthetic derivative in 0·5 N acetic acid, to which a trace of Adam's platinum oxide had been added, were hydrogenated using a hydrogen pressure of 50 lb./sq.in. in a stainless-steel bomb with constant shaking. No derivative remained after 15 min. as judged by the colourless nature of the solutions. The acid conditions used greatly reduced the time stated as necessary for complete reaction by Towers *et al.* (1954), who worked in neutral solution. The solutions were evaporated to dryness, and the residues redissolved in 100 μl. portions of distilled water. Each solution (20 μl.) was applied as spots to each of four chromatograms, together with marker spots containing suitable mixtures of known amino acids including γ-methylglutamic acid. The chromatograms were developed in water-saturated phenol, the *n*-butanol–NH_3 solvent above, collidine–lutidine mixture (Dent, 1947), and the *n*-butanol–acetic acid mixture of Partridge (1948), and the amino acids detected after ninhydrin spraying. Both the unknown and synthetic derivatives yielded a well marked spot of an amino acid inseparable from γ-methylglutamic acid in all four solvent systems, together with a trace of another amino acid identified as γ-amino-α-methylbutyric acid by chromatographic comparison with a sample of this amino acid prepared by hydrogenation of γ-amino-α-methylenebutyric acid, isolated by Fowden & Done (1953*b*). This non-α-amino acid presumably arose by slight decarboxylation during the reduction of the DNPH derivatives, and this may be compared with a similar behaviour observed for the derivatives of other dicarboxylic keto acids (Towers *et al.* 1954). We have observed that there is a tendency for decarboxylation to occur more rapidly, with the production of increased amounts of ω-amino acids, as the pH of the solution subjected to reduction is raised.

Visible absorption spectra. The absorption spectra of equimolar solutions of the two derivatives in N-NaOH, and of a similar solution of the DNPH derivative of α-oxoglutaric acid, were determined over the wavelength range 380–700 mμ. using a Unicam SP. 500 spectrophotometer (Fig. 2).

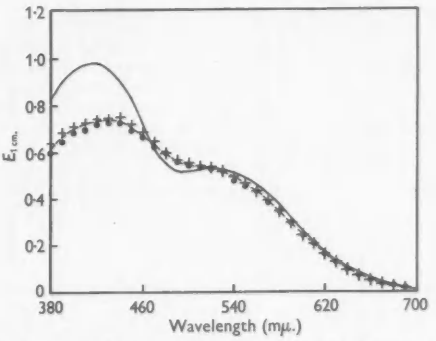


Fig. 2. Reproduction of the visible absorption spectra of the DPNH derivatives of keto acids; 0·005% (w/v) in N-NaOH. ——, derivative of α-oxoglutaric acid; +---+, derivative of the biologically synthesized γ-methylene-α-oxoglutaric acid; ●- -●, derivative of the unknown keto acid investigated.

Those of the unknown and the derivative of γ-methylene-α-oxoglutaric acid are seen to be virtually identical, but they differ from that of the α-oxoglutaric acid derivative.

Infrared spectra. The infrared absorption spectra of Nujol mulls of the two derivatives were compared (Fig. 3). It will be seen that the two spectra possess all the same main absorption frequencies, and only differ very slightly in the relative intensities of these absorption peaks.

X-ray-diffraction spectra. The two derivatives in the form of their ammonium salts were subjected to comparative X-ray-diffraction analysis. The diffraction lines obtained from the unknown were more diffuse than those obtained from the synthetic derivative, but the two spectra appeared to be essentially identical. When the unknown and synthetic derivatives were compared as free acids, their diffraction patterns differed, presumably due to differences in the crystalline forms of the two derivatives.

Oxidative degradation. Trial experiments showed that the DNPH derivative of α-oxoglutaric acid could be oxidized to succinic acid (the expected product) by H_2O_2. The α-oxoglutaric acid derivative (1 mg.) was treated with 100 μl. of 30% (w/v) H_2O_2 soln. at 100° for 20 min., and the remaining solution chromatographed on a sheet of Whatman no. 2 filter-paper strip against marker spots of α-oxoglutaric and succinic acids containing 100 μg. of each acid. The lower organic layer of the two-phase mixture produced after equilibration of 80 ml. $CHCl_3$, 80 ml. *tert.*-amyl alcohol, 80 ml. water and 30 ml. 98–100% (w/v) formic acid was used as the developing solvent (L. E. Bentley, unpublished), and after complete removal of the solvent, the chromatogram was evenly sprayed with a 60% (v/v) ethanolic solution of bromocresol green adjusted to pH 5·5. The positions of the organic acids were revealed as yellow areas on a greenish blue background. The only detectable organic acid produced during the oxidation flowed to a position identical with that of succinic acid (R_F 0·63); the spot of α-oxoglutaric acid had an R_F value of 0·46.

The same conditions were used to oxidize the unknown and the derivative of γ-methylene-α-oxoglutaric acid, which, if identical, should both yield malonic acid. The latter acid was added as an additional marker to the chromatograms developed in the same solvent. The only oxidation product detected in either oxidized solution was an organic acid moving at the same R_F value (0·54) as that determined for malonic acid.

In order to establish that the DNPH derivative of γ-methyl-α-oxoglutaric acid did not give malonic acid as the main oxidation product under these conditions, a small amount of the derivative was prepared and isolated in a similar manner to that already described for γ-methylene-α-oxoglutaric acid, by using 5 mg. of γ-methylglutamic acid as the starting material in the transamination reaction. On oxidation of the derivative of γ-methyl-α-oxoglutaric acid with H_2O_2, an organic acid chromatographically identical with methylsuccinic acid (R_F 0·77) appeared as the main oxidation product.

DISCUSSION

From the literature it appears that γ-methylene-α-oxoglutaric acid is a new keto acid that has not been chemically synthesized. In order to obtain the DNPH derivative of the keto acid, we had therefore to resort to a biological synthetic method, using γ-methyleneglutamic acid as the starting material. The recent chemical synthesis of this amino acid (Wailes *et al.* 1954) has established its structure,

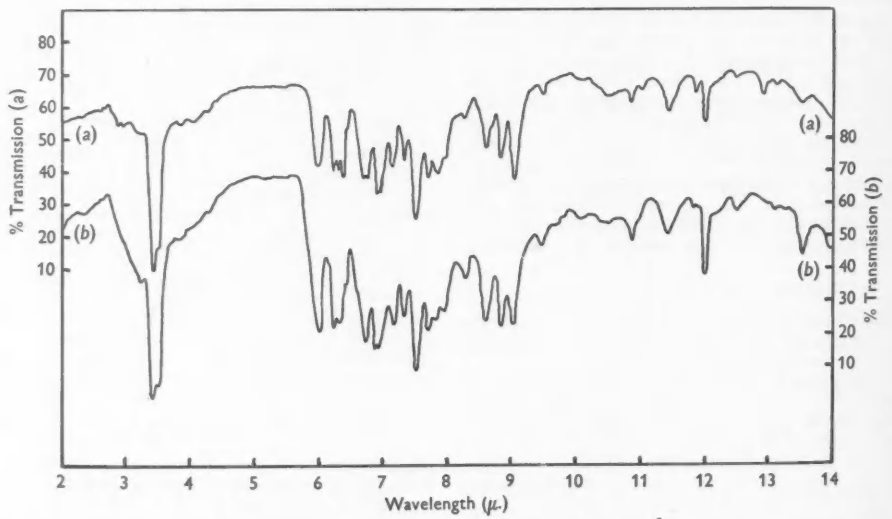


Fig. 3. Reproduction of the infrared absorption spectra of Nujol mulls of (*a*) the DPNH derivative of γ-methylene-α-oxoglutaric acid, and (*b*) the derivative of the unknown keto acid investigated.

whilst many detailed investigations of the transamination reaction have established its mechanism as firmly as those of most chemical syntheses. There can therefore be little doubt concerning the structure of the biologically synthesized keto acid, unless enzymic reduction of the —C=C— bond had also occurred.

The evidence obtained by hydrogenation and oxidative degradation, coupled with our general experience with groundnut plants, in which we have never obtained any evidence for the natural occurrence of the saturated γ-methyl derivative of glutamic acid or of enzymes capable of reducing the γ-methylene derivative, proved that the biological synthesis did in fact yield the unsaturated γ-methylene-α-oxoglutaric acid. The identity of the DNPH derivative of this keto acid with the material isolated from the groundnut seedlings is firmly established by the variety of the comparative methods used.

Although Towers & Steward (1954) consider their substance *B* to be the 2:4-dinitrophenylhydrazone of γ-methylene-α-oxoglutaric acid, they record a much higher m.p. (268–270° decomp.) for their material than we have observed for either the derivative of the natural keto acid (228°) or the biologically synthesized acid (224°). The reason for this discrepancy is not immediately obvious, for although geometrical isomerism is possible in DNPH derivatives, it seems unlikely that such isomers would differ widely in their m.p.'s. We consider that we have presented the stronger evidence in support of the exact nature of our isolated derivative, and note that our recorded m.p.'s (224–228°) are of the order that might be predicted from those recorded for other DNPH derivatives of α-keto dicarboxylic acids (oxalacetic acid, 211°; α-oxoglutaric acid, 219°; from El Hawary & Thompson, 1953).

Seedlings, rather than mature plants, were extracted because they could be grown and handled more easily. Earlier analyses (Fowden, 1954) had also demonstrated that a very rapid production of γ-methyleneglutamine and γ-methyleneglutamic acid accompanied the process of germination, and a similarly rapid accumulation of γ-methylene-α-oxoglutaric acid seemed likely. Further experiments are in progress to determine the rates of production of this acid and other keto acids during seedling growth, and their distribution in the different organs of more mature plants.

Provided the process used for the extraction of the DNPH derivative of γ-methylene-α-oxoglutaric acid from the seedlings was reasonably efficient, the yield (12 mg. from 2 kg. fresh weight) suggests that the difficulties likely to be encountered in the isolation of useful amounts of the free acid from seedlings would be very great. These facts emphasize the need for a suitable chemical method of synthesis of the keto acid, giving amounts that would be of use in studying its metabolism in the plant. The low concentrations of this and other oxo acids found in seedlings are probably indicative of their rapid rates of turnover, and it seems plausible that γ-methylene-α-oxoglutaric acid is an intermediate stage in the synthesis of γ-methyleneglutamic acid from carbohydrate or lipid storage materials, being converted into the amino acid either by transamination or directly by reductive amination with ammonia.

SUMMARY

1. The keto acids present in extracts of groundnut seedlings (*Arachis hypogaea*) were investigated and characterized by paper chromatography of their 2:4-dinitrophenylhydrazine (DPNH) derivatives. α-Oxoglutaric, glyoxylic and pyruvic acids were identified, whilst comparable amounts of a new keto acid were also present in the seedlings.

2. Using cellulose-powder column and paper-chromatographic procedures, 12 mg. of the DNPH derivative of the new keto acid were isolated from 2 kg. of seedlings.

3. Using a variety of physical and chemical methods, the DNPH derivative of the new keto acid was shown to be identical with the corresponding derivative of γ-methylene-α-oxoglutaric acid

$$(HOOC.C(:CH_2).CH_2.CO.COOH)$$

prepared by biological transamination from the analogous amino acid.

4. The implications of the presence of the new keto acid in the seedlings are discussed.

We wish to thank Professor Kathleen Lonsdale, F.R.S., and Dr L. J. Bellamy for arranging the X-ray and infrared spectra comparisons respectively, Dr L. E. Bentley for supplying unpublished information regarding the solvent used for organic acid chromatography, and Dr J. A. Elvidge for a gift of methylsuccinic acid. We are also indebted to the Royal Society for a grant in aid of apparatus, whilst one of us (J. A. W.) expresses gratitude to the Agricultural Research Council for a personal maintenance grant.

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The Estimation of Thiols and Disulphides by Potentiometric Titration with Silver Nitrate

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This paper describes further developments in the estimation of thiols by potentiometric titration with silver nitrate. A previous paper (Cecil, 1950) described a method in which the thiols were allowed to react with a known excess of silver nitrate followed by back-titration with potassium bromide. Although the results then obtained agreed well with the theoretical figures, it has now been shown that the agreement was due to compensation of errors.

It has been found that these errors can be avoided by titrating the thiols directly with the silver nitrate in buffered solutions. The silver mercaptides formed tend, in most cases, to form complexes with additional silver ion which can interfere with the determination of the thiol end point. However, the stability of these complexes varies greatly with pH, and a range of pH has been found for every compound in which the thiol group can be titrated satisfactorily.

As the pH is raised, so the concentration of silver ion in equilibrium with free thiol falls and becomes so low that reproducible potentials cannot be obtained with a silver electrode. This difficulty has been overcome by the development of the silver thiol electrode (Cecil, 1954) which is reversible to the thiol as well as to the silver ion.

As in the previous work, the disulphides are first allowed to react with sodium sulphite and the thiol formed estimated in the same way.

THEORETICAL

Reaction of thiols with silver nitrate. The reaction of thiols with silver nitrate is described by the equations

$$RS^- + Ag^+ \rightleftharpoons RSAg, \quad (1)$$

$$RS^- + H^+ \rightleftharpoons RSH. \quad (2)$$

In the presence of solid silver mercaptide

$$S = [Ag^+] \times [RS^-], \quad (3)$$

where S is defined as the solubility product. The equilibrium equation for (2) is

$$K = \frac{[RS^-] \times [H^+]}{[RSH]}, \quad (4)$$

K being the dissociation constant of the thiol. This may be expressed in terms of total thiol concentration, c, where $c = [RSH] + [RS^-]$ so that (4) now becomes

$$[RS^-] = c.K/(K + [H^+]), \quad (5)$$

and substituting in (3)

$$S = [Ag^+]\, c.K/(K + H^+). \quad (6)$$

The end-point titre (c_{ep}) is reached when the total silver added equals the total thiol originally present:

$$[Ag^+_{ep}] + [RSAg] = c_{ep} + [RSAg],$$

or

$$[Ag^+_{ep}] = c_{ep}. \quad (7)$$

From (6) the silver ion concentration at the end point is

$$\left.\begin{aligned} [Ag^+_{ep}] &= \left(S\,\frac{K + [H^+]}{K}\right)^{\frac{1}{2}} \\ &= \sqrt{S'}, \end{aligned}\right\} \quad (8)$$

where S' is the apparent solubility product under any one set of conditions.

This equation is of limited application at present because of the difficulties of defining the various quantities. K is known only for thiols that do not contain amino groups. Different ionic species of the same compound are likely to have different solubility products and these must be taken into account when using eqn. 6. Lastly, species other than thiol, for example sulphite and silver mercaptides, bind silver ion sufficiently to depress the end-point concentration below the predicted value. To this extent therefore the method is empirical, and sufficient of each titration curve must be plotted to determine the end-point potential.

If there is to be a simple relation between $[Ag^+]$ and c during a titration, then the term $K/(K + [H^+])$ must remain constant. Adequate buffering is therefore essential. Because of the low silver ion concentration prevailing in the presence of thiol, the usual buffer salts acetate, phosphate, borate, as well as nitric or sulphuric acid, may be used, provided their concentrations are kept low.

The titrations are followed potentiometrically with silver or silver thiol electrodes. At pH <2·5 the silver ion concentrations are sufficiently high to be followed with a silver electrode. At higher pH this is not so, and a silver thiol electrode must be used. This consists of a silver electrode covered with a thin layer of silver mercaptide.

In the presence of thiol the reaction on the electrode surface is

$$RS^- + Ag \rightleftharpoons RSAg + e, \quad (9)$$

and the potential is described by the equation

$$E_{RS^-} = E^\circ_{RS^-} - \frac{RT}{F} \ln [RS^-], \quad (10)$$

and substituting for $[RS^-]$ from eqn. 5

$$E_{RS^-} = E^\circ_{RS^-} - \frac{RT}{F} \ln c + \frac{RT}{F} \ln \frac{(K + [H^+])}{K}. \quad (11)$$

In the presence of silver ion the potential is described by the equation

$$E_{Ag^+} = E^\circ_{Ag^+} + \frac{RT}{F} \ln [Ag^+]. \quad (12)$$

In the presence of both thiol and silver ion, as the electrode can have but one equilibrium potential, this must be described equally by (11) and (12), i.e. $E_{RS^-} = E_{Ag^+} = E$.

It is convenient to use one standard potential only, so combining equations (6) and (12)

$$E = E^\circ_{Ag^+} - \frac{RT}{F} \ln c + \frac{RT}{F} \ln S + \frac{RT}{F} \ln \left(\frac{K + [H^+]}{H^+} \right), \quad (13)$$

and from eqns. 11 and 13

$$E^\circ_{RS^-} = E^\circ_{Ag^+} + \frac{RT}{F} \ln S. \quad (14)$$

The potentials obtained with a silver thiol electrode may be interpreted in terms of either thiol or silver ion concentrations, whichever is convenient. The titration curves obtained may be treated in the same way as those obtained with silver electrodes.

It was reported earlier (Cecil, 1954) that thiols containing free amino groups show anomalous electrode responses at high pH. This effect may be ignored in the present work because (*a*) the effect is small at concentrations below 10^{-4} M and so will not affect the titration curves near the end point, and (*b*) the effect is small at pH <8 and the titrations described are normally done below this pH.

Reaction of disulphides with sodium sulphite

The reactions are

$$RSSR + SO_3^{2-} \rightleftharpoons RS^- + RSSO_3^-, \quad (15)$$

$$RS^- + H^+ \rightleftharpoons RSH, \quad (2)$$

$$SO_3^{2-} + H^+ \rightleftharpoons HSO_3^-. \quad (16)$$

Although the precise conditions of reaction, i.e. time, temperature and SO_3^{2-} concentration, have to be determined for each compound, there are two points of general application which define the pH range. First, Stricks & Kolthoff (1951) have shown that reaction (15) is reversible, the extent of the back reaction depending on the amount of RS^- formed. Thus if the reaction is to go to completion the reaction pH must be sufficiently below the pK of the thiol. This effect is particularly important where the thiol formed must be titrated at a pH lower than that used for reaction.

Secondly, the present authors (Cecil & McPhee, 1954) have found from kinetic studies that the bisulphite ion does not react with any of these disulphides. The second pK of sulphurous acid is 7·2 (Tartar & Garretson, 1941) and so the rate of reaction will fall off rapidly with pH.

EXPERIMENTAL

Apparatus

The apparatus used for the titrations was the same as described previously (Cecil, 1950) with minor alterations, as follows:

(*a*) Stirring was effected by mounting the titration vessel on a rotating platform driven by an air-turbine directly beneath (Stock & Fill, 1950). The turbine was worked by suction from a filter pump. This method of stirring is efficient and, unlike the bubbling method previously used, does not affect the readings of the electrodes.

(*b*) The reference half-cell was Ag/AgCl instead of Hg/Hg_2Cl_2—this simplified the apparatus and was equally satisfactory. The 3·5 M-KCl was saturated with solid AgCl and a silver wire immersed in it. The difference in potentials caused by this change was +35 mv.

Silver electrodes

(*a*) *Construction.* 28 s.w.g. platinum wire was sealed into a glass tube so that 5–10 mm. was exposed. The end of the wire was fused so as to eliminate sharp edges. In use the whole of the exposed part of the wire was immersed in the solution with the glass tube covering the wire at the air–water interface. 36 s.w.g. tinned copper wire was used for leads. Soldered joints on platinum are unsatisfactory and so a short piece of silver wire was fused to the platinum and the copper lead soldered to it. Mercury must not be used for making contact as it penetrates the glass seal and causes large errors in potential.

(*b*) *Gold plating.* Platinum is slowly attacked by thiols, even through a silver coating, and to prevent this a protective coating of gold was used. This too is attacked slowly but can be stripped off whenever the electrode is re-silvered. A potassium aurocyanide solution was used (Clarke, 1928) with a current density of 2–3 mA/cm.^{2} for approximately 5 min. with stirring.

(*c*) *Silver plating.* A 'soft' deposit of silver was found to be most satisfactory. This was obtained by using a potassium argentocyanide solution with a high carbonate content, together with a high current density (Sanigar, 1929). The current density was 15–30 mA/cm.^{2} and the composition of the plating solution was as follows: $AgNO_3$, 3·5 g.; KCN, 4·0 g. (=50% excess); K_2CO_3, 10 g.; water, 100 g. The plating time is not critical, 10–60 sec. being satisfactory. After plating, the electrodes were washed thoroughly in distilled water.

Silver thiol electrodes

(*a*) H_2S *treatment.* Silver electrodes were dipped into aqueous H_2S (0·01 to 0·005 M) for 10–60 sec., to give a thin, even coating of Ag_2S. This should not produce any visible tarnishing of the electrode surface. If the sulphide layer is too thick, equilibrium will be slow and the potentials will drift when the stirring is started and stopped.

Solutions of H_2S oxidize rapidly. If degassed water is used the solutions keep much longer but the H_2S attacks the

silver too rapidly. It was found that the CO_2 normally present in water slows the action of H_2S on silver. The most satisfactory method is to dissolve the H_2S in degassed water but, before use, to mix this solution with water containing some dissolved CO_2.

(*b*) *Equilibration with thiol.* This is an ion-exchange process between S^{2-} and the thiol concerned. It is rapid with RS^- and slow with RSH. Two concentrations of thiol were made up, approx. 10^{-3} and 10^{-4} M respectively, buffered in $Na_2B_4O_7$ at pH $\simeq$ 9. The electrodes were immersed in each solution alternately until reproducible potentials were obtained. Fresh solutions were used for each immersion: three immersions were usually sufficient. If the thiol was not available, a disulphide–sulphite mixture was used instead.

(*c*) *Cleaning of electrodes.* The silver layer was removed with conc. HNO_3, and the gold layer with aqueous Cl_2 or by anodizing in HCl. This should leave the platinum bright and smooth. If not, it should be boiled in conc. HNO_3 for 10 min.

The preparation of these electrodes has been described in detail because they are the key to the whole method. The time required for plating and equilibration is not excessive and, once prepared, electrodes have been used for over a hundred titrations. Differences of potential between different electrodes do occur, and are reflected in differences in end-point potential. The titration end points, however, are not affected. The reason for these differences is still under investigation but they are generally due to attack of the gold or platinum by the thiol.

Reagents

β-Mercaptopropionic acid (L. Light and Co. Ltd., Poyle Trading Estate, Colnbrook, Bucks). A sample was dried over anhydrous $MgSO_4$ and distilled *in vacuo*. The capillary in the distillation flask was fed with dry, O_2-free nitrogen. B.p. 58–60° at approximately 1 mm. Hg.

L-*Cysteine hydrochloride* (Roche Products Ltd., Welwyn Garden City, Herts). The sample was kept in a desiccator over $CaCl_2$.

L-*Cysteine*. Prepared from the hydrochloride by the method of du Vigneaud, Audrieth & Loring (1930).

DL-*Homocysteine* (L. Light and Co. Ltd.). A sample was sublimed at 180° at 0·02 mm. Hg.

Homocysteine thiolactone hydrochloride. Prepared from homocysteine by the method of Riegel & du Vigneaud (1936).

Reduced glutathione (GSH). A sample, DCB 524, was kindly supplied by the Distillers Company (Biochemicals) Ltd., Fleming Road, Speke, Liverpool 19.

ββ′-Dithiodipropionic acid. Prepared from β-mercaptopropionic acid by oxidation with I_2. After crystallization from water it had m.p. 156° (uncorr.). Biilmann (1905) gives 155–156° (uncorr.).

L-*Cystine* (Roche Products Ltd.). Recrystallized once from hot dil. HCl.

Diformylcystine. Prepared from L-cystine by the method of Fruton & Clarke (1934). Potentiometric titration with NaOH gave an equiv. wt. of 146, theoretical = 148.

Diacetylcystine diethyl ester (diacetylcystine ester). Cystine ester dihydrochloride was prepared by the method of Abderhalden & Wybert (1916). The ester (0·2 g.) was dissolved in 2 ml. sat. aq. $NaHCO_3$ and extracted 3 times with 7 ml. portions of ethyl acetate. The combined extracts were dried over anhydrous Na_2SO_4 and filtered. A stream of ketene was passed through the solution at 20°. The ethyl acetate was evaporated *in vacuo* and a white crystalline product remained which melted sharply at 124° (corr.). Yield 60%. Hollander & du Vigneaud (1931) give m.p. 124·5° (corr.).

DL-*Homocystine* (L. Light and Co. Ltd.). A sample was dissolved in hot dilute HCl and a little Norite added. The solution was filtered, and the homocystine precipitated by adjustment to pH 5. The precipitate was filtered and washed with water. The purification was repeated once.

Oxidized glutathione (GSSG). Prepared from GSH by the method of Schöberl & Hornung (1938).

Sodium sulphite. 0·1 M solutions of reagent grade Na_2SO_3 containing 2×10^{-3} M ethylenediaminetetraacetic acid (EDTA) were made up each week. The EDTA greatly reduced the rate of oxidation both of the Na_2SO_3 and of the thiols in contact with it. The halide content, measured as described by Cecil (1950), must not exceed 10^{-3} moles/mole of Na_2SO_3.

Nitric acid. 0·1 N solutions were prepared as described by Cecil (1950).

Silver nitrate. Reagent grade $AgNO_3$ was recrystallized once from water.

Buffer solutions. The following substances of reagent grade were used; KH_2PO_4, K_2HPO_4, $Na_2B_4O_7$, H_2SO_4, sodium acetate, and acetic acid. Solutions of pH > 5 contained EDTA to the extent of one mole/50 moles of buffer. The following solution was used extensively and is referred to as phosphate *A*; KH_2PO_4, 0·05 M; K_2HPO_4, 0·05 M; EDTA, 2×10^{-3} M. Molarities quoted refer to the phosphate radical.

Distilled water. Glass-distilled water was used throughout.

METHOD

Solutions of thiols were made up in freshly degassed water and kept at 2° when not in use. The rate of oxidation was then 1–5% in 24 hr. for a 10^{-3} M solution, depending on the compound and its freedom from heavy metal impurities. Cystine and homocystine were dissolved in 0·01 N-H_2SO_4. The other disulphides were dissolved in water.

The reaction of disulphides with Na_2SO_3 was carried out either at room temperature or at 60 ± 5°. At room temperature open test tubes or titration vessels could be used without appreciable oxidation occurring during the time needed for reaction. At 60° the rate of oxidation is greater and Thunberg tubes were used. These were flushed with O_2-free nitrogen 3 times and finally evacuated. Since the Na_2SO_3 was present in excess it also served to control the pH, if necessary with the addition of a little HNO_3.

Samples (1 or 2 ml.) of the thiol solutions were taken and the buffer was added. The total concentration of buffer should not normally exceed 0·05 M or the electrodes may be slow in reaching equilibrium. Disulphide–sulphite mixtures were treated in the same way. 0·1 N-$AgNO_3$ was used for titration and the volume added plotted against potential. The end points were taken as the mid-points of the titration curves, determined graphically, and were reproducible to ± 0·05 μl. of 0·1 N-$AgNO_3$.

pH was measured with a glass electrode calibrated as recommended in British Standard (1950) 1647.

Table 1. *Titration of thiols with* $AgNO_3$

β-Mercaptopropionic acid and GSH titrations were carried out with sufficient 0·1 N-HNO_3 or 0·1 N-H_2SO_4 added to bring pH to 1·8–2·2. Cysteine and homocysteine titrations were done at pH 6·5–7·0 using phosphate *A*, *ca.* 0·02 M.

Substance	Concn. range used (M)	pH range in which the thiol can be titrated without error	Titres expressed as % of the weighed concn.
β-mercaptopropionic acid	*ca.* 10^{-3}	1·5–4·5	98
GSH	5×10^{-5} to 6×10^{-3}	1·8–2·2	99·5
Cysteine	5×10^{-5} to 10^{-3}	2·5–7·5	99
Homocysteine	*ca.* 10^{-3}	3·0–9·0	97

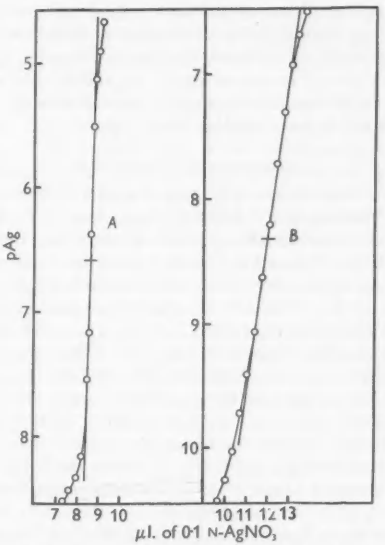

Fig. 1. *A*: titration curve of 1 ml. of $8·6 \times 10^{-4}$ M β-mercaptopropionic acid (in 0·02 N-HNO_3) with 0·1 N-$AgNO_3$ at pH 1·80. *B*: titration curve of 1 ml. of the same solution at pH 6·72 (0·02 M phosphate *A*). The apparent molarity is $1·16 \times 10^{-3}$, an error of +35 %. The electrode potentials have been expressed in pAg units. 1 pAg unit = 58 mv at 18°.

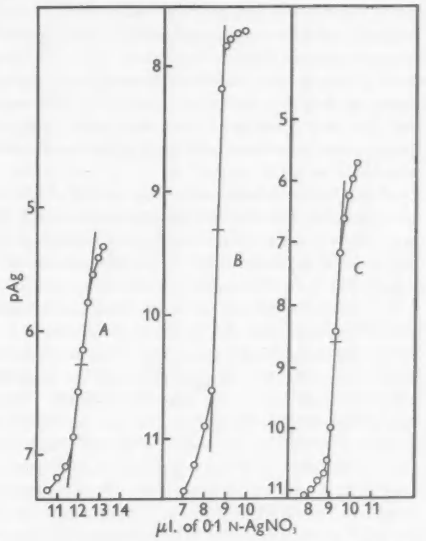

Fig. 2. *A*: titration curve of $1·21 \times 10^{-3}$ M GSH with $AgNO_3$ at pH 1·85. *B*: titration curve of $8·5 \times 10^{-4}$ M cysteine hydrochloride at pH 6·75. *C*: titration curve of $9·2 \times 10^{-4}$ M homocysteine at pH 6·75. Owing to the steepness of this curve the pAg scale has been halved. The electrode potentials have been expressed in pAg units. 1 pAg unit = 58 mv at 18°.

RESULTS

Estimation of thiols

The range of pH in which the different thiols can be titrated with silver nitrate varies considerably. For routine purposes it is convenient to standardize conditions as far as possible. The compounds described here fall into two groups, namely those titrated in acid solution (pH 1·8–2·2) using a silver electrode, and those titrated in neutral solution at pH 6·5–7·0 using the appropriate silver thiol electrode. The buffers used were sulphuric acid or nitric acid in the acid range and phosphate *A* in the neutral range, all in a concentration of 0·01 to 0·02 M.

These results are summarized in Table 1; column 3 gives the full pH range over which each thiol can be titrated without error. The figures given in column 4 for the percentage recovery are each the mean of at least ten determinations. Repeated determinations on the same solutions agreed within ± 0·5 % of the mean.

β-Mercaptopropionic acid. This may be titrated without error between pH 1·5 and 4·5 (Fig. 1*A*). At higher pH the curves become flatter owing to silver complex formation, and more than one equivalent of silver is bound. At pH 6–7 the error is +35 % (Fig. 1*B*).

GSH. GSAg complexes with silver ion over a wide range of pH and GSH can only be titrated satisfactorily between pH 1·8 and 2·2 (Fig. 2*A*). At pH > 2·5 the curves are asymmetrical and difficult

to interpret. At pH 1·7 and below, the curves become flatter and an additional 12% of silver is bound. Comparison of the present results with those obtained previously (Cecil, 1950) show that the bromide back-titration method gives results which are 4% high over the concentration range 5×10^{-5}M to 6×10^{-3}M.

Cysteine and cysteine hydrochloride. Free cysteine may be titrated from pH 3·5 to 7·5. At pH > 7·5 the titration curves become very flat and the end points cannot be measured. At pH < 3·5 apparently normal curves are obtained, but the amount of silver bound increases as the pH falls, until at pH < 2·5 it reaches a constant amount 17% greater than the expected value. This factor of +17% was found to be constant over a wide range of concentration, namely 5×10^{-5}M to 1×10^{-3}M. The same factor of +17% is found over the same range of concentration, if the bromide back-titration method (Cecil, 1950) is used.

Cysteine hydrochloride must be titrated at pH > 6 or the chloride interferes. The presence of 10^{-3}M cystine does not interfere. The upper halves of the cysteine (and cystine) titration curves are abnormally flat (Fig. 2*B*). The reason for this is not known but the steepness of the curves is such that it does not interfere with the end-point determination.

Homocysteine. This can be titrated without interference due to complex formation between pH 3·0 and 9·0. The curves are slightly asymmetrical but they are so steep that this is of no consequence (Fig. 2*C*). The silver homocysteine electrodes are rather slow in reaching equilibrium in phosphate *A* at pH 6·5 to 7·0. Equilibrium is reached more quickly in a sulphite buffer at the same pH and, provided the amount of any disulphide present is known, this buffer is preferable.

The behaviour in acid solution is curious. At pH 1·66–2·5 in nitric acid the titres of silver nitrate are 1–2% high and no precipitate of mercaptide is formed. If sulphuric acid is used, a precipitate is formed and the titre is 5% high at pH 1·92 and 17% high at pH 1·67.

In view of the possibility that this behaviour might be due to the formation of the thiolactone the following experiments were tried. First, a sample of homocysteine thiolactone hydrochloride was titrated with silver nitrate at pH ≃ 2 and gave a titration for chloride only. The thiolactone itself did not react with the silver ion. Since the concentration of the free silver ion in a homocysteine titration is less than in a chloride titration, homocysteine can be estimated in the presence of its thiolactone without interference.

Secondly, two samples of homocysteine in nitric acid at pH ≃ 2 were titrated, one immediately, and one after standing for 1 hr. at room temperature. There was no reduction in the titre on standing, showing that the rate of formation of thiolactone under these conditions is negligible. Equally, if the behaviour of homocysteine in sulphuric acid were due to thiolactone formation, the titre would be expected to be low rather than high.

Estimation of disulphides

The conditions required for the sulphite–disulphide reaction fall into three groups. (*a*) Cystine, homocystine and diacetylcystine ester react rapidly at pH 6·5–7·0, and the thiols formed can be titrated at the same pH. These substances can therefore be titrated with silver nitrate immediately after adding the sodium sulphite. (*b*) Diformylcystine can also be titrated at the pH of reaction with sulphite, but the reaction is slow and must be allowed to proceed to completion before titration. (*c*) GSSG reacts rapidly with sulphite, while dithiodipropionic acid reacts slowly. Both, however, must be brought to pH 1·8–2·2 before titration, and the reaction must be complete before acidification. These results are summarized in Table 2.

N-Acetylcysteine ester and *N*-formylcysteine have not been studied as pure substances but only as sulphite–disulphide reaction mixtures. *N*-Acetylcysteine ester can be titrated with silver nitrate from pH 1·5 to 9·0 without any sign of silver complex formation. The titre falls off above pH 7·5 because of the reversibility of the reaction with sulphite. *N*-Formylcysteine may be titrated from pH 6·5 to 9·0, though the reverse reaction with sulphite becomes significant above pH 8·0. Below

Table 2. *Estimation of disulphides. Summary of results*

The temperature of reaction was 60° for diformylcystine and dithiodipropionic acid. The other compounds were allowed to react at room temperature.

	Reaction conditions with Na_2SO_3				
Substance	Moles of Na_2SO_3/mole of RSSR	pH	Time allowed for reaction (min.)	pH for titration	Titres expressed as % of the weighed concn.
Cystine	15–25	6·5–7·0	0	6·5–7·0	100
Homocystine	15–25	6·5–7·0	0	6·5–7·0	99
Diacetylcystine ester	15–25	6·5–7·0	0	6·5–7·0	100
Diformylcystine	25–30	7·0–7·5	15	7·0–7·5	98
GSSG	15–25	6·5–7·0	30	1·8–2·2	98
Dithiodipropionic acid	50–60	7·5–8·0	30	1·8–2·2	98

pH 6·5, complexing with silver commences. The error is +15% at pH 4–5, falling to +4% at pH≃2. *N*-Formyl cysteine is unusual in that it forms a silver mercaptide, which remains in solution under conditions which give a satisfactory titration.

Effect of chloride

If a mixture of two anions, both of which form insoluble silver salts, is titrated with silver nitrate, the jump in electrode potential corresponding to the end point of the less soluble anion will occur early, and its titre will be low. The size of the error depends on the difference in solubility products of the salts and the relative concentration of the two anions. A quantitative treatment is given by Kolthoff & Furman (1949).

In the estimation of GSSG and dithiodipropionic acid, mixtures of each disulphide with sodium sulphite are titrated with silver nitrate at pH≃2. At this pH the apparent solubility products of the two silver mercaptides (S' of eqn. 8) are sufficiently close to that of silver chloride for small errors to arise if the sodium sulphite contains too much chloride as impurity. Thus, using approximately 10^{-3}M thiol solutions, GSH shows an error of −1% with 0·1 equivalent of chloride, and −4% with 0·5 equivalent. Similarly, β mercaptopropionic acid shows an error of −2% with 0·5 equivalent of chloride. These figures have been used in setting the limits for the chloride content of sodium sulphite given earlier.

The effect on the titrations at pH 6·5–7·0, using silver thiol electrodes, is less. Using approximately 10^{-3}M cysteine, there is no error with 3 equivalents of chloride, but with 5 equivalents the electrode becomes very sluggish and end points cannot be determined. This is probably because, under these conditions, the electrode potential is controlled by the thiol rather than the silver ion and, as the concentration of chloride is raised, it tends to displace the thiol from the surface of the electrode.

DISCUSSION

The range of pH in which the different silver mercaptides form stable complexes with silver nitrate is interesting, since it throws light on the groups involved. β-Mercaptopropionic acid complexes at pH > 4·5 but not below this pH. 1·02 equivalents of silver are bound at pH 4·6, and this rises to 1·35 at pH 6·7. The carboxyl pK is 4·32 (Borsook, Ellis & Huffman, 1937) and so it seems that the —COO^- form has a greater tendency to bind silver than the —COOH form. GSH and cysteine show an opposite effect. At pH < 1·7 GSH binds 1·12 equivalents of silver and at pH < 2·5 cysteine binds 1·17 equivalents. The carboxyl pK values of GSH are 2·12 and 3·53 (Pirie & Pinhey, 1929) and that of cysteine is 1·36 (Cannan & Knight, 1927). Although there is no precise correspondence, in these compounds the change from —COO^- to —COOH must be responsible for complex formation. Homocysteine shows the same tendency to complex in acid solution, but this tendency depends on the acid used. The reason for this is not known, but it is unlikely to be due to formation of the thiolactone, since this substance does not react appreciably with small amounts of silver ion at pH≃2.

The carboxyl pK of *N*-formylcysteine does not appear to have been measured but is likely to be slightly below that of formylglycine, given as 3·4 by Zief & Edsall (1937). There is no complex formation between pH 6·5 and 9·0. At pH 4·7, however, the amount of silver bound rises to 1·15 equivalents and then falls to 1·04 equivalents at pH≃2. It appears therefore that maximum complex formation occurs when the —COO^- and —COOH forms are both present, though once again there is no precise correspondence with the carboxyl pK. The behaviour of cysteine and GSH at high pH is complex and is still being investigated.

The kinetics of the sulphite–disulphide reaction have been reported briefly (Cecil & McPhee, 1954) and will be given in detail in a later paper.

It remains to compare the present results with those obtained previously by the bromide back-titration method. In the latter method a known excess of silver nitrate was added to the thiol solution at pH≃2 and the excess measured by a potentiometric titration with potassium bromide. It depended on the basic assumption that, at this pH, any silver not bound by thiol was titratable by bromide. The agreement with the theoretical figures apparently indicated that this was so. One of the difficulties, however, in developing a method for the analysis of thiols is the lack of standards. Thiols are not easy substances to purify and they oxidize easily. In fact the GSH used in the previous work has since been shown to be slightly impure. Consequently, analysis by back titration, which gives an error of +4%, agreed well with the theoretical figures. The original sample of GSSG was made from this sample of GSH and so was subject to the same error.

Cystine was allowed to react with sulphite, acidified, and the cysteine formed estimated by back titration, apparently satisfactory results being obtained. It was not known then that the sulphite reaction is reversible at high pH and in fact it was carried out at approximately pH 8·5. Consequently, only 0·82–0·85 of an equivalent of cysteine was formed, but this fact was masked by the cysteine titration error of +17%. These errors have been shown to be constant over a wide range of concentration and so it is possible to allow for them. The back-titration method was used (Cecil, 1950) to

follow the reaction of GSSG and cystine with silver nitrate. Since GSSG and GSH were both subject to the same error the interpretation of this reaction is unaffected. The cystine concentrations on the other hand were correct, whereas the cysteine figures were 17% high. This reaction is therefore slower than it appeared and the difference in reactivity between the two substances greater. Details will not be given here, as work on these and other compounds is continuing.

SUMMARY

1. A method for the estimation of four thiols by potentiometric titration with silver nitrate is described. The titrations are carried out in buffered solutions, the pH used depending on the particular compound. In all cases one of two pH ranges, 1·8–2·2 or 6·5–7·0, can be used.

2. At pH < 2·5 the silver ion concentrations are sufficiently high for the titrations to be followed with a silver electrode.

3. At pH > 2·5 the silver ion concentrations are too low to be followed with a silver electrode and the appropriate silver thiol electrode must be used. The preparation of these electrodes is described and a simple thermodynamic treatment is given.

4. The method is applied to the estimation of six disulphides. These are first allowed to react with sodium sulphite to give one equivalent of the corresponding thiol. The factors governing the optimum pH for the reaction are discussed and the conditions for each compound are given.

5. The conditions under which the different silver mercaptides form stable complexes with additional silver are discussed.

6. This work disclosed errors in previous work on the estimation of thiols and disulphides. These are discussed and the corrections given.

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Complex-Formation between Polypeptides and Metals

1. APPLICATION OF VARIOUS EXPERIMENTAL METHODS TO THE GLYCINE–COPPER SYSTEM

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In recent years complex-formation between amino acids and various metals has been extensively studied with the aid of potentiometric titration (Albert, 1950, 1952; Monk, 1951; Perkins, 1952). The theory and practical application of the method has been fully described by Albert (1950), and the results obtained are summarized in the form of stability constants which for a simple ligand L^- (such as an amino acid anion) and a divalent metal M are the equilibrium constants for the reactions

$$M^{2+} + L^- \rightleftharpoons ML^+, \quad (1)$$

$$ML^+ + L^- \rightleftharpoons ML_2, \quad (2)$$

so that $K_1 = [ML^+]/[M^{2+}]\,[L^-]$

and $K_2 = [ML_2]/[ML^+]\,[L^-]$.

The constants K_1 and K_2 may be combined in the expression $K_1K_2 = K_s$, where K_s is the equilibrium constant for the overall reaction between the metallic ions and the amino acid, i.e.

$$M^{2+} + 2L^- = ML_2.$$

When the method of potentiometric titration was applied to the study of complex-formation between cupric ions and the simplest polypeptide, glycylglycine, it became evident that the situation was essentially more complicated than with an amino acid such as glycine. With glycine in the zwitterion form one proton is liberated for each molecule of amino acid taken up by the metal. A preliminary consideration of the titration curve of glycylglycine in the presence of cupric chloride suggests that in this case at least two protons are liberated for every peptide zwitterion which reacts with the metal.

It seemed desirable, therefore, in order to obtain a confirmed interpretation of metal-complex formation in such a system to supplement the potentiometric titration data with information provided by other techniques. Those tested were copper-electrode potential measurements, electrophoresis, and spectrophotometric observations in the visible range of the spectrum. It seemed necessary in the first instance to test the value of these methods by applying them to the interpretation of the relatively well-understood copper–glycine system. This paper describes how these methods were applied and demonstrates that the results obtained from their use are consistent with the view that glycine reacts with copper according to eqns. 1 and 2 above.

EXPERIMENTAL

Materials

The copper salt used was A.R. $CuCl_2, 2H_2O$. The glycine was supplied by British Drug Houses Ltd. and found to be chromatographically pure. Before use it was dried *in vacuo* at 78°.

Methods

Potentiometric titrations. Three solutions were examined containing $CuCl_2, 2H_2O$ (0·04262 g.) and glycine in the molar ratio of 1:1, 1:2 and 1:6, respectively. The reagents in each case were dissolved initially in such a volume of boiled-out distilled water that the total concentration of copper at the mid-point of each titration was almost exactly 0·005 M.

The solution to be titrated was contained in a 150 ml. beaker fitted with a rubber bung which held a glass electrode, a saturated calomel electrode, a thermometer and a capillary glass tube through which N_2 was passed continuously for stirring and maintaining an inert atmosphere. Two additional small holes were made in the bung; one for insertion of the burette tip and the other (lined with a piece of glass tubing) for insertion of a copper electrode or for a fine pipette through which samples of the solution might be withdrawn for spectrophotometric analysis.

The alkali used for titration was carbonate-free 0·1 N-KOH standardized against weighed portions of potassium hydrogen phthalate and stored in a waxed bottle from which it was delivered by a 2 ml. burette with drawn-out tip. The alkali was added in a series of small portions each of which generally contained 0·1 equiv. KOH/molecule of chelating agent present in the solution. After each addition of alkali, the pH of the solution was determined with a glass electrode and a Beckman Model *G* pH meter. All the experiments were done at 20°.

If we define $\bar{n}$ as the average number of ligand molecules bound by each metal ion at any stage in the process of complex-formation (Bjerrum, 1941) and $[L^-]$ as the corresponding concentration of free chelating species, then, provided that certain conditions are satisfied, K_1 and K_2 can be calculated from eqns. 3 and 4 without actual measurement of the free metal-ion concentration being made (Albert, 1950):

$$K_1 = \frac{\bar{n}}{(1-\bar{n})[L^-]}, \quad (3)$$

$$K_2 = \frac{(\bar{n}-1)}{(2-\bar{n})[L^-]}, \quad (4)$$

$[L^-]$ may be evaluated from the equation

$$\beta[L^-] = [L_0] - [B^+] - [H^+], \quad (5)$$

where

$$\beta = \frac{2[H^+]^2}{K_a K_{a'}} + \frac{[H^+]}{K_a},$$

K_a (the ionization constant of the NH_3^+ group) $= [L^-][H^+]/[^+HL^-]$; $K_{a'}$ (the ionization constant of the COOH group) $= [^+HL^-][H^+]/[^+HLH]$, $[L_0]$ = total concentration of amino acid and $[B^+]$ = the concentration of base added to the solution at any point. The corresponding value of $\bar{n}$ may be found from

$$\bar{n}[M] = [L_0] - \alpha[L^-], \quad (6)$$

where $\alpha = [H^+]^2/K_aK_{a'} + [H^+]/K_a + 1$ and $[M_0]$ = total concentration of metal.

In applying these equations we have used concentrations instead of activities since all solutions examined were sufficiently dilute to ensure that the activity coefficient of every ionic species was nearly unity. The problem of obtaining the best values of the constants K_1 and K_2 has been considered by Irving & Rossotti (1953). This method, though valuable in the simpler cases, would not seem to be readily applicable to the more complex situations considered in the following papers of this series and so is not employed here. It is doubtful whether the improvements in the values which would be obtained by such arithmetical refinements would be of practical significance in work of the present type.

When values for K_1 and K_2 have thus been estimated at restricted points on the titration curve of glycine in the presence of cupric ions, their validity throughout the entire process of complex-formation may be tested by calculating the theoretical titration curve which they imply for the copper–glycine system and comparing this with the experimental curve.

If $[L_0]/[M_0]$, the ratio of the molar concentrations of ligand and metal in a solution, is denoted by X, then at any pH the concentration $[L^-]$ of free chelating anions in this solution is given by

$$K_1K_2\alpha[L^-]^3 - K_1K_2(X-2)[M_0][L^-]^2 + K_1\alpha[L^-]^2 - K_1(X-1)[M_0][L^-] + \alpha[L^-] = X[M_0]. \quad (7)$$

The value of L^- found from this equation may then be inserted in eqn. 5 to obtain the amount of base required to bring the solution to the chosen pH.

Copper-electrode potential measurements. The potential of a copper electrode, partially immersed in a solution containing cupric ions, may be read against a calomel electrode using a suitable potentiometer in which the null point is detected by a high-resistance valve voltmeter. The current taken by our instrument was of the order 10^{-11} A, so that while a reading was being taken, polarization of the electrode was reduced to a minimum.

The electrodes used were lengths of copper wire of about 1 mm. in diameter. Before use, an electrode was washed in N-NH_3 followed by distilled water. It was then electroplated in a solution containing 15% (w/v) $CuSO_4$, $5H_2O$ + 5% (w/v) H_2SO_4. When covered with an even film of copper, the wire was rinsed in distilled water and placed in the titration vessel. After each pH reading had been taken during a titration, a measurement of copper-electrode potential was made. When K_1 and K_2 have been calculated from the potentiometric titration data, $[M^{2+}]$ may be found at any pH from the equation

$$[M^{2+}] = [M_0]\,(1 + K_1[L^-] + K_1K_2[L^-]^2).$$

The theoretical electrode potential corresponding to any value of $[M^{2+}]$ may then be calculated using the equation

$$E = E^0 - \frac{RT}{zF} \ln c,$$

where E^0 = standard potential of copper electrode and c = concentration of cupric ions, and the observation that in an 0·001 M solution of pure $CuCl_2$, $2H_2O$ the potential of a copper electrode read against our saturated calomel electrode was 0 mv at 20°. The use of the above equation was subject to the provision that the activity coefficient of the copper in our solution should be practically unity. The copper-electrode potential values obtained experimentally were compared with those calculated from these equations.

Electrophoresis. A drop of a solution containing $CuCl_2$ and glycine at any pH, when placed on a filter-paper strip moistened with glycine solution of equal concentration and pH to whose ends a potential is applied, will move according to the effective mean charge on the metal at that pH. However, in order to determine the displacement of the spot due to electrophoresis alone it is necessary to take into account the behaviour of the spot due to electroendosmosis and adsorption of the filter paper. The extent of movement due to electroendosmosis may be measured by placing initially alongside the test spot a drop of a solution containing molecules which are uncharged and have no tendency to be adsorbed on the paper. A suitable marker is glucose. The displacement of this marker will then be due to electroendosmosis alone.

Any tendency for the test spot to be adsorbed on the paper may be determined by comparing its movement with that of the marker substance when no current is passed and the liquid phase is allowed to move slowly along the paper due to a siphoning effect alone.

Strips of Whatman no. 54 chromatographic filter paper 5 cm. in width were used. At two points on a line perpendicular to the long axis of the filter paper were placed respectively a 5 μl. drop of the copper–glycine solution being examined and, 2·5 cm. away, a 5 μl. drop of a 2% (w/v) glucose solution as marker of the electroendosmotic flow. It was thought desirable in any experiment to apply the drops on a line whose distance from the mid-point of the strip would be approximately equal to that of the mean position of the spots detected at the end. When the spots applied to a strip were dry, the strip was wetted in a solution of glycine equal in concentration to that of the test spot and at the same pH, care being taken not to move the spots. The wet strip was then placed between two pairs of glass plates in such a way that the central part of the paper, free of the plates, was tautly suspended while the last few cm. at each end dipped down into glass troughs containing some of the glycine solution used to wet the paper. The glass plates were held between two large glass plates resting on a block. The sides of the small chamber in which the central part of the paper was suspended were closed by insertion of suitable pieces of thick glass plate. A piece of platinum foil was partially immersed in the solution in each trough and the potential between these electrodes had a gradient of about 5 v/cm. along the paper strip.

When the current had been passed for 5 hr. the papers were removed and quickly dried in a horizontal position by a current of hot air. Each strip was then divided lengthwise. The half containing the copper–glycine spot was sprayed with an aqueous 0·1% solution of sodium diethyldithiocarbamate. Reaction between the copper and this substance led to the immediate appearance of a light-brown spot. The other half was sprayed with a 3% solution of *p*-anisidine hydrochloride in *n*-butanol and dried for 10–15 min. at about 110° when the glucose, which gave a brown spot, was seen to have moved towards the cathode.

In order to find whether the copper–glycine solution was adsorbed on the filter paper, similar experiments were done in which no current was passed and glycine solution was put in only one trough of each pair. The liquid then flowed slowly over the paper towards the empty trough. After the same period of 5 hr. the papers were dried and sprayed as before.

When the resultant electrophoretic movement of the copper in a particular copper–glycine solution at any pH has been measured experimentally, it can be compared with that predicted from consideration of the expected distribution of the metal as free ions and glycine complexes. These forms exist in the ratio

$$[M^{2+}]:[ML^+]:[ML_2] = 1:K_1[L^-]:K_1K_2[L^-]^2 \qquad (8)$$

at any pH, the copper gradually losing its positive charge as complex-formation proceeds.

Spectrophotometric observations. Visible changes in the colour of a copper–glycine solution during titration with alkali may be attributed to the formation of successive complexes having different absorption spectra. If these spectra were known and also the relative amounts in which the complexes are present at any pH value it would be possible to calculate the composite absorption curve for the solution at that pH assuming that the spectral contributions of the various complexes are additive. Absorption curves thus calculated could then be compared with those observed spectrophotometrically.

The experimental observations were made with a Unicam SP. 500 spectrophotometer, a sample of each solution being withdrawn at intervals during the course of titration, then replaced in the titration vessel after its optical density had been measured at 10 mμ. intervals throughout the visible range of the spectrum.

By using the equilibrium constants obtained from the potentiometric titration experiments, the distribution of the metal in a solution at any pH is found from expression (8) above.

The absorption coefficients at various wavelengths to be attributed to the free cupric ion were obtained directly by spectrophotometric observations on a solution containing 0·005 M-$CuCl_2$. Those of the complex ML_2 were taken as a first approximation to be identical with the absorption spectrum of the solution containing cupric ions and glycine in the ratio of 1:6 at a pH value in the neighbourhood of 10 where practically all of the copper is present in this form.

If we now consider the experimentally observed absorption curve of the solution containing copper and glycine in the molar ratio of 1:1 or 1:2 at a pH where the species ML^+ is predominant, we can derive the absorption curve of this complex using those of M^{2+} and ML_2 already obtained and the calculated ratio in which these three species exist.

Thus if M^{2+}, ML^+ and ML_2 are present at a particular pH in the amounts α_1, α_2 and α_3 respectively, and their absorption coefficients at a given wavelength are E_1, E_2 and E_3, respectively, then $E_0 = \alpha_1 E_1 + \alpha_2 E_2 + \alpha_3 E_3$ is the observed absorption of the solution at that pH. E_2 may then be found by substitution of the values known for the other quantities in the equation. This process may be repeated until the absorption coefficient of ML^+ has been calculated for every λ at which spectrophotometric examination of the solution has been made.

These absorption coefficients have been derived to fit the experimentally observed absorption curve of a particular solution at one pH. Consequently, slight adjustments may have to be made to them and to the absorption coefficients chosen for ML_2 in order that they combine with those of M^{2+} at all pH values to give the best possible agreement with the observed absorption curves of all three solutions examined.

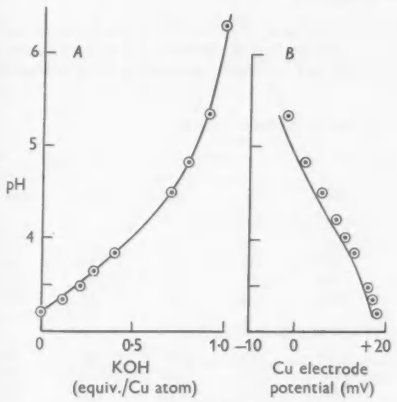

Fig. 1. *A*, titration curve of solution containing 18·76 mg. glycine (0·005 M) + 42·63 mg. $CuCl_2, 2H_2O$ (0·005 M) in 48·75 ml. *B*, change with pH in potential of copper electrode immersed in above solution. ——, observed; ⊙, calculated.

RESULTS

The values calculated here for the ionization constants of the amino and carboxyl groups of glycine at 20° are $K_a = 10^{-9\cdot86}$ and $K_{a'} = 10^{-2\cdot22}$ at concentrations of 0·01 and 0·1 M respectively. These values are the same as those given by Albert (1950).

From the potentiometric titration data of the 1:1 and 2:1 glycine–copper solutions, the values

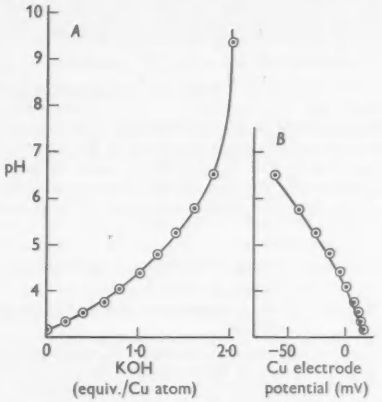

Fig. 2. *A*, titration curve of solution containing 37·52 mg. glycine (0·01 M) + 42·63 mg. $CuCl_2, 2H_2O$ (0·005 M) in 47·5 ml. *B*, change with pH in potential of copper electrode immersed in above solution. ——, observed; ⊙, calculated.

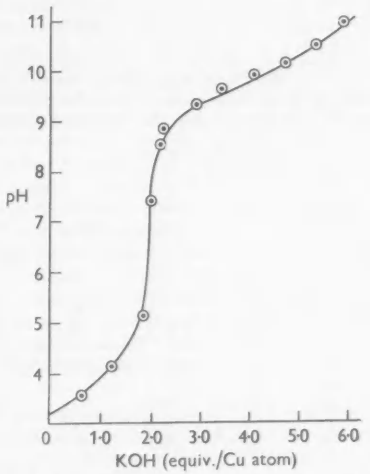

Fig. 3. Titration curve of solution containing 112·57 mg. glycine (0·03 M) + 42·63 mg. $CuCl_2, 2H_2O$ (0·005 M) in 42·5 ml. ——, observed; ⊙, calculated.

calculated for the stability constants of the copper–glycine complexes are $K_1 = 10^{8\cdot5}$ and $K_2 = 10^{7\cdot0}$. These combine to give $K_s = 10^{15\cdot5}$ which is the value reported by Albert (1950). By the use of these values of K_1 and K_2 the expected titration curves of solutions containing $CuCl_2$ and glycine in the molar ratios of 1:1, 1:2 and 1:6 and also the copper-electrode potential expected at various points in these titrations were calculated. In Figs. 1–3 these theoretical curves are compared with those observed. The titration of the solution containing equimolar quantities of $CuCl_2$ and glycine was not continued above pH 6, since a blue gelatinous precipitate, presumably $Cu(OH)_2$, appears about this stage. This precipitation is due to the fact that reaction between glycine and cupric ions does not end with the formation of the complex ML^+ even when the reagents are present in equimolar concentration. Some of the dichelate ML_2 complex exists also in equilibrium with free cupric ions, some of which will be precipitated as $Cu(OH)_2$ when the pH of the solution rises above 6.

The results of the electrophoresis experiments with solutions containing $CuCl_2$ and glycine in the

Table 1. *Electrophoretic behaviour of copper–glycine complexes*

Change with pH in the electrophoretic movement of the copper in a solution containing 0·005 M-$CuCl_2$ + 0·01 M glycine. Figures given are distances traversed by the copper–glycine and glucose spots on a filter-paper strip to which a potential of 5 v/cm. was applied. Corrections are made for electroendosmosis and adsorption. All distances are measured as positive in the direction of the cathode. The theoretical change with pH in charge on metal is shown by distribution of metal in form of free ions and glycine complexes. G^- represents the glycine anion.

pH	3·0	5·0	8·0	10·0
Average observed movement of copper–glycine spot (cm.)	9·7	5·3	4·7	4·4
Average observed movement of glucose spot (cm.)	3·9	4·2	5·0	4·4
Calculated movement of copper–glycine spot due to electroendosmosis (glucose movement × R_F of copper–glycine spot)	3·3	3·4	4·7	4·4
Net movement of copper–glycine spot due to electrophoresis	6·4	1·9	0·0	0·0
Distribution of copper (%) Cu^{2+}	79·0	4·6	—	—
Distribution of copper (%) CuG^+	20·8	65·0	3·8	0·6
Distribution of copper (%) CuG_2	0·2	30·4	96·2	99·4

Table 2. *Copper–glycine complexes*

A: distribution of copper in form of cupric ions and copper–glycine complexes at various pH values in solutions containing 0·005 M-$CuCl_2$, $2H_2O$ and different concentrations of glycine. *B*: comparison of observed and calculated optical densities of the solutions at various pH values. The figures are $\log I_0/I \times 10^3$ and have been adjusted to refer to a solution in which $[Cu_0] = 0{\cdot}005$ M at each pH. G^- represents the glycine anion.

	A. Distribution of metal as			*B*. Optical densities at (mμ.)							
				600		650		700		750	
pH	Cu^{2+} (%)	CuG^+ (%)	CuG_2 (%)	Obs.	Calc.	Obs.	Calc.	Obs.	Calc.	Obs.	Calc.
				(*a*) Glycine concn., 0·005 M (composition of solution as in Fig. 1)							
3·49	69·8	29·8	0·4	—	—	49	49	73	72	86	84
4·22	40·4	57·0	2·6	—	—	81	83	108	108	115	114
5·33	18·5	72·2	9·3	64	68	109	114	136	136	135	133
6·30	14·0	73·3	12·7	72	75	120	122	140	141	136	136
				(*b*) Glycine concn., 0·01 M (composition of solution as in Fig. 2)							
3·35	58·6	40·5	0·9	—	—	62	62	88	86	97	96
4·41	13·7	73·3	13·0	74	76	122	122	141	142	135	136
5·25	2·5	56·0	41·5	122	124	160	161	155	156	130	131
6·51	0·1	19·0	80·9	181	181	201	201	158	159	108	109
				(*c*) Glycine concn., 0·03 M (composition of solution as in Fig. 3)							
4·14	4·7	65·4	29·9	110	106	152	155	156	151	135	135
8·84	—	—	100·0	208	209	217	219	159	159	97	97
9·60	—	—	100·0	208	209	220	219	161	159	99	97

molar ratio of 1:2 are given in Table 1. These show that the positive charge on the metal is gradually reduced in magnitude with increase in pH.

The distribution of the metal in the form of free ions and these complexes at various pH values in the three solutions examined are given in Table 2*A*. By means of these figures and the absorption curves of the species given in Fig. 4, the optical density expected for each solution at several pH values was calculated. In Table 2*B* a selection of these figures is compared with the experimental observations.

DISCUSSION

The purpose of this paper is chiefly to describe, test and justify, methods for use in studying the formation of complexes of Cu^{2+} with simple polypeptides. On potentiometric titration little comment is required except to say that the results agree closely with those in the literature. Observations have been made not only with the usual 2:1 glycine–$CuCl_2$ molar concentration ratio but also with 1:1 and 6:1 ratios. The agreement of the theory with the experimental data has been shown by what we consider the best method in such investigations, namely by calculating the titration curve of the solution from the values of K_1 and K_2 and comparing this with the experimentally observed curve. This is of importance in dealing with more complicated systems since the error of the experimental observations may be approximately estimated, while that of derived constants cannot be so readily found.

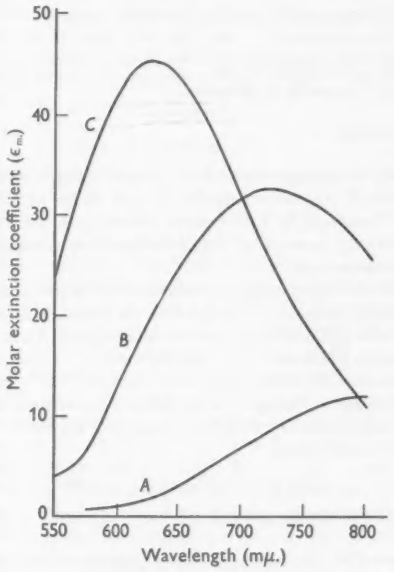

Fig. 4. Computed absorption curves of free cupric ions and copper–glycine complexes. Curve *A*, Cu^{2+}; curve *B*, CuG^+ and curve *C*, CuG_2. (G^- represents the glycine anion.)

Our experience with the copper electrode suggests that this type of electrode is of only limited value due to the experimental difficulties involved in its use. In particular, it was difficult to ensure stability of the electrode especially when the cupric-ion concentration was low. Below a cupric-ion concentration of about 10^{-7} M the electrode readings became quite unreliable. It is to be remembered that measurements in solutions of low cupric-ion concentration will be complicated by the reaction $Cu^{2+} + Cu \rightleftharpoons 2Cu^+$ which takes place at the electrode. This reaction has an equilibrium constant

$$K = [Cu^+]^2/[Cu^{2+}] \simeq 10^{-6}.$$

Because of this it has been found by Tourky & Wakkad (1948) that a copper electrode immersed in a solution of pure cupric sulphate will give reliable indication of the cupric-ion activity only when this actually exceeds 10^{-4}. However, in solutions containing copper–glycine complexes the concentration of Cu^{2+} at any pH will be kept relatively constant, since even if some Cu^{2+} is reduced to Cu^+ in the vicinity of the electrode, the Cu^{2+} concentration will be replenished by dissociation of the complexes. This buffering action may account for the comparatively satisfactory readings of the electrode in our solutions down to a cupric-ion concentration of about 10^{-7} M.

The electrophoresis experiments indicate that the change with pH in the sign and magnitude of the resultant charge on the metal in a solution containing copper and glycine in the ratio of 1:2 corresponds well with the degree of metal chelation as calculated from the values found for K_1 and K_2. It is also important to note that there is no apparent reversal of the sign of the charge on the metal, for in the next paper it will be shown that such a reversal is in fact observed with copper and glycylglycine in alkaline solution.

Very good agreement is obtained between the optical density measurements made on each of the three solutions over a range of pH and the figures calculated by combining the characteristic absorption curves of M^{2+}, ML^+ and ML_2 in proportions calculated from a knowledge of the values of K_1 and K_2.

The degree of reproducibility of the observed sets of figures, and the sensitivity of the theoretical sets of figures to changes in the values taken for K_1 and K_2, make the agreement between the two sets a reliable check on the validity of the interpretation of the process of complex formation in this system.

The method of spectrophotometric analysis is therefore considered the best of the three methods used here for testing the results derived from potentiometric titration experiments. It proved to be of particular value in a later study of the combination between cupric ions and diglycylglycine, where the reaction constants of the system could not be evaluated from the titration data alone.

SUMMARY

1. The following methods have been studied with a view to their application to the reactions between metals and polypeptides in aqueous solution: (*a*) potentiometric titration, (*b*) copper-electrode potential measurements, (*c*) electrophoresis, and (*d*) spectrophotometric observations.

2. When these methods were applied to the study of complex formation between glycine and cupric ions they confirmed the view that two complexes ML^+ and ML_2 are formed.

3. Even in alkaline solution and with an excess of glycine there was no evidence from the electrophoretic observations of the formation of a complex with a negative charge such as would be found if one cupric ion combined with more than two glycine anions.

4. Absorption curves in the visible range of the spectrum have been calculated for the copper–glycine complexes.

We wish to acknowledge a maintenance grant from the Department of Scientific and Industrial Research to one of us (H. D.) and to thank Professor A. Albert of the National University of Australia for help in developing the potentiometric titration technique.

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Complex-Formation between Polypeptides and Metals

2. THE REACTION BETWEEN CUPRIC IONS AND SOME DIPEPTIDES

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The reaction of cupric ions with peptides has been studied qualitatively by a number of workers, often with the object of shedding light on the biuret reaction. Kober & Sugiura (1912) described the colours produced on the addition of alkali to solutions containing cupric ions along with a di-, tri- or tetra-peptide in the metal:peptide molar ratio of 1:1, and tried to correlate these colours with the formation of bonds between the metal and nitrogen atoms. This work was extended by the spectrophotometric investigations of Kober & Haw (1916) with various peptides, some of considerable complexity. More recently, information on the absorption coefficients of the copper complexes of di- to octa-peptides has been supplied by Plekhan, Gavrilov & Poddubnaya (1942), Plekhan & Gavrilov (1948) and Plekhan (1951). These workers, and also Rising, Parker & Gaston (1934) and Wenaas (1937), who isolated crystalline copper complexes of tri- and tetra-glycylglycine, respectively, postulated the existence (in a copper–peptide complex) of links between the metal and the atoms of the peptide bond. Confirmation of the existence of this linkage in metal–peptide complexes has been obtained by Chouteau & Lenormant (1951) and Chouteau (1951) by means of infrared spectrophotometric measurements.

Monk (1951) studied the effect of the presence of glycylglycine on the solubility of cupric iodate at various pH values. On the assumption that the observed increase in solubility is due to complex-formation between the cupric ions and the peptide and that two complexes are formed as with glycine, he calculated the following stability constants for these complexes:

$$K_1 = [CuG^+]/[Cu^{2+}]\,[G^-] = 10^{6\cdot0}$$

and

$$K_2 = [CuG_2]/[CuG^+]\,[G^-] = 10^{5\cdot6},$$

where G^- represents the glycylglycine anion. These results were confirmed by potentiometric-titration experiments. The solutions studied in this work contained an excess of peptide and were apparently examined in the pH range 4–8.

In the present work the reaction between cupric ions and each of the dipeptides glycylglycine, glycyl-L-leucine, glycyl-L-tyrosine and carnosine (β-alanylhistidine) has been investigated. Complex-formation between cupric ions and glycylglycine has been the most comprehensively studied using the methods described in the previous paper (Dobbie, Kermack & Lees, 1955). Since it appears that in this case the conclusions reached by the method of potentiometric titration are confirmed by the observations made using the other experimental methods, only the former method has been used to study complex-formation involving the other three dipeptides.

EXPERIMENTAL

Materials

The dipeptides were obtained from Roche Products Ltd. and were found to be chromatographically pure, each giving a single spot when run one-dimensionally on Whatman no. 4 paper with (*a*) phenol–water (4:1, w/v), (*b*) phenol–*m*-cresol–water (2:2:1, w/v/v), or (*c*) *n*-butanol–acetic acid–water (4:1:5, by vol.) as solvent.

Methods

The experimental methods used in this work have been described in the previous paper (Dobbie *et al.* 1955) on the combination between cupric ions and glycine. The modified mathematical treatment of the potentiometric-titration data necessary for each peptide will be given in the appropriate sections.

It was necessary to evaluate the ionization constants of the peptides used. This was done by potentiometric titration of peptide solutions with 0·1N-KOH or N-HCl, values for the constants being calculated as described by Albert (1950).

RESULTS

Glycylglycine

The values calculated for the ionization constants of this peptide are $K_a = 10^{-8.37}$ and $K_{a'} = 10^{-3.12}$. Those recorded in the literature are $10^{-8.23}$ and $10^{-3.19}$ (Glasstone & Hammel, 1941).

Potentiometric titrations were done on solutions containing 0·005M-$CuCl_2$, and glycylglycine in the molar ratios of 1:1, 1:2, 1:3 and 1:4. The titration curves obtained are shown in Figs. 1–4, respectively. Inspection of these curves reveals that when a solution containing equimolar quantities of $CuCl_2$ (0·005M) and glycylglycine is titrated with alkali, one equivalent of alkali/copper atom is required to raise the pH of the solution from 3·89 to 4·73, and a second to raise it to 7·95. From this it may be inferred that two protons are being liberated in the range 3·89–7·95. The first of these must be associated with the combination of a cupric ion and a peptide anion to give the complex CuG^+ (where G^- represents the glycylglycine anion), while the second may be released from the peptide bond of that complex due to the metal becoming bound to the nitrogen or oxygen atom there. Alternatively, the buffering between pH 4·73 and 7·95 may be due to the uptake of a hydroxyl group by the metal in the complex CuG^+. These two explanations will be discussed more fully later on, but meanwhile it may be observed that in a mathematical treatment of the situation, the loss of a proton from the complex CuG^+ with ionization constant K_c is identical with the uptake of a hydroxyl group with constant $K_b = K_w/K_c$. Each leads to the formation of a neutral complex.

During the addition of a further equivalent of alkali above pH 7·95 the copper in the complex CuG is presumably taking up an OH^- group.

The titration curve of a solution containing $CuCl_2$ and glycylglycine in the ratio of 1:2 shows that four protons/copper atom are released during complex-formation. The loss of the first two, between pH values 3·89 and 4·46 and pH values 4·46 and 6·00, respectively, is presumably due to the formation of the complexes CuG^+ and CuG already mentioned, while that of the third between pH 6·00 and 9·00 may be due to the uptake of a peptide anion by the complex CuG to give CuG_2^-. The ionization of this last complex to give CuG_2^{2-} would then account for the fourth proton liberated. If the second and fourth reactions postulated here (namely the formation of CuG and CuG_2^{2-}) involve the displacement of peptide-bond protons by the copper, then the outcome of combination between cupric ions and glycylglycine will be the stable dichelate complex shown in Fig. 5.

During the titrations of solutions containing 3 and 4 peptide molecules/copper atom, 5 and 6 protons/copper atom are released respectively. These observations suggest that two peptide molecules are bound by each metal ion with the loss of four protons, while any additional peptide molecule loses the ionizable proton of its NH_3^+ group in accordance with the pK_a of 8·37.

On the assumption that combination between cupric ions and glycylglycine involves the following steps:

$$Cu^{2+} + G^- \rightleftharpoons CuG^+,$$
$$CuG^+ \rightleftharpoons CuG + H^+,$$
$$CuG + H_2O \rightleftharpoons CuG(OH)^- + H^+,$$
$$CuG + G^- \rightleftharpoons CuG_2^-,$$
$$CuG_2^- \rightleftharpoons CuG_2^{2-} + H^+,$$

where G^- represents the glycylglycine anion, mathematical equations may be derived to obtain, from the potentiometric-titration data, values for the following equilibrium constants:

$$K_1 = [CuG^+]/[Cu^{2+}]\,[G^-],$$
$$K_2 = [CuG_2^-]/[CuG]\,[G^-],$$
$$K_c = [CuG]\,[H^+]/[CuG^+],$$
$$K_{c'} = [CuG(OH)^-]\,[H^+]/[CuG],$$
$$K_{c''} = [CuG_2^{2-}]\,[H^+]/[CuG_2^-].$$

As in the case of combination between cupric ions and glycine, the stability constants K_1 and K_2 may each be expressed at certain stages of the complexing process in terms of $[L^-]$ the concentration of free ligand anions, and $\bar{n}$ the corresponding average number of ligand anions bound per metal ion.

During the addition of the first two equivalents of alkali (BOH) per metal atom to a solution containing peptide of concentration $[L_0]$ and metal of concentration $[M_0]$, $[L^-]$ is given by:

$$\beta[L^-] = \gamma[L_0] - [B^+] - [H^+], \qquad (1)$$

where $$\gamma = \frac{1 + 2K_c/[H^+]}{1 + K_c/[H^+]}$$

and

$$\beta = (\gamma + 1)\,[H^+]^2/K_a K_{a'} + \gamma[H^+]/K_a + \gamma - 1.$$

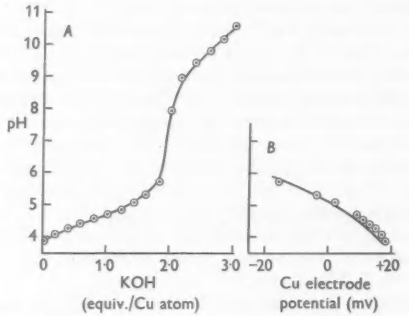

Fig. 1. *A*, titration curve of solution containing 33·02 mg. glycylglycine (0·005 M) + 42·63 mg. $CuCl_2, 2H_2O$ (0·005 M) in 46 ml. *B*, change with pH in potential of copper electrode immersed in above solution. ——, observed; ⊙, calculated.

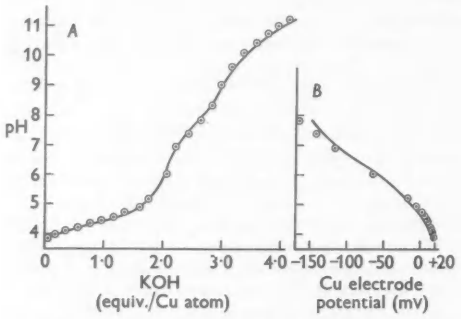

Fig. 2. *A*, titration curve of solution containing 66·04 mg. glycylglycine (0·01 M) + 42·63 mg. $CuCl_2, 2H_2O$ (0·005 M) in 45 ml. *B*, change with pH in potential of copper electrode immersed in above solution. ——, observed; ⊙, calculated.

The corresponding value of $\bar{n}$ is given by

$$\bar{n}[M_0] = [L_0] - \alpha[L^-], \qquad (2)$$

where $\alpha = [H^+]^2/K_a K_{a'} + [H^+]/K_a + 1$,

and hence K_1 may be evaluated from

$$K_1 = \frac{\bar{n}}{(1-\bar{n})\,[L^-]} \cdot \frac{1}{1 + K_c/[H^+]}. \qquad (3)$$

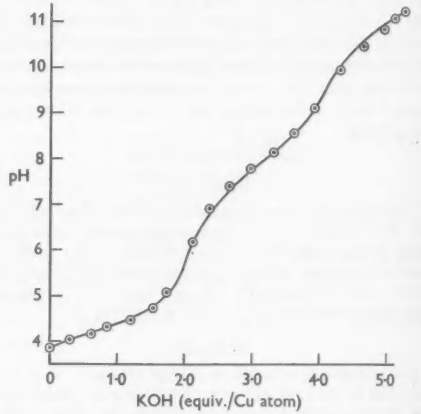

Fig. 3. Titration curve of solution containing 99·06 mg. glycylglycine (0·015 M) + 42·63 mg. $CuCl_2, 2H_2O$ (0·005 M) in 42·5 ml. ——, observed; ⊙, calculated.

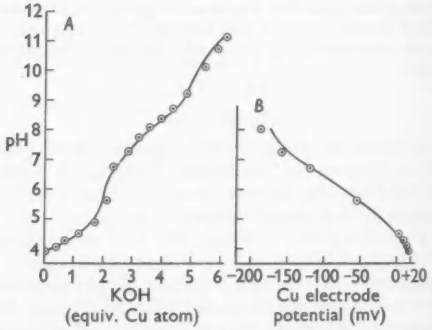

Fig. 4. Titration curve of solution containing 132·08 mg. glycylglycine (0·020 M) + 42·63 mg. $CuCl_2, 2H_2O$ (0·005 M) in 42·5 ml. ——, observed; ⊙, calculated.

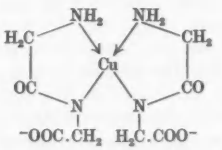

Fig. 5. Proposed structural formula for the copper-glycylglycine complex CuG_2^{2-}. (G^- represents the glycylglycine anion.)

During the addition of further equivalents of alkali to a solution in which $[L_0] > [M_0]$, $[L^-]$ may be found from

$$\beta'[L^-] = \delta[L_0] + (2-\delta)[M_0] - [B^+] + [OH^-], \quad (4)$$

where $$\delta = \frac{1 + 2K_{c''}/[H^+]}{1 + K_{c''}/[H^+]}$$

and $$\beta' = (\delta+1)[H^+]^2/K_aK_{a'} + \delta[H^+]/K_a + \delta - 1,$$

and $\bar{n}$ is given by eqn. 2; hence K_2 may be calculated from

$$K_2 = \frac{(\bar{n}-1)}{(2-\bar{n})[L^-]} \frac{1}{1 + K_{c'}/[H^+]}. \quad (5)$$

Eqns. 1 and 4 each contain two unknowns, and a method of trial and error was adopted to evaluate $[L^-]$ throughout any titration.

Values for K_c and $K_{c''}$, which were suggested by inspection of the titration data, were tried in eqns. 1 and 3 and eqns. 4 and 5, respectively. Small successive adjustments were then made to K_c and $K_{c''}$ until values were found which gave the most constant sets of values for K_1 and K_2 when calculated at several points on the titration curve.

For a solution containing equimolar quantities of $CuCl_2$ and glycylglycine, the constant $K_{c'}$ must also be evaluated. The pH of the solution when 2·5 equivalents of alkali have been added is taken as a provisional value of $pK_{c'}$.

When approximate values have thus been obtained for all the equilibrium constants, the expected titration curve of a solution containing $CuCl_2$ and glycylglycine in any ratio may be calculated.

The value of $\bar{n}$ at any pH at which it is expected to be less than 1 is given by

$$\frac{(1-\bar{n})(X-\bar{n})}{\bar{n}} = \frac{\alpha}{K_1[M_0](1 + K_c/[H^+])},$$

where $$X = [L_0]/[M_0].$$

The corresponding value of $[L^-]$ may be calculated from eqn. 2 and the amount of base required to reach the chosen pH from eqn. 1. For a pH at which $\bar{n}$ is expected to be greater than 1, the value of $\bar{n}$ may be found from

$$\frac{(2-\bar{n})(X-\bar{n})}{(\bar{n}-1)} = \frac{\alpha}{K_2[M_0]} \frac{1}{1 + K_{c''}/[H^+]},$$

and the corresponding values of $[L^-]$ and of $[B^+]$ from eqns. 2 and 4, respectively. In the neighbourhood of $\bar{n} = 1$, the accuracy of any value of $[L^-]$ found as above requires to be checked by insertion in the equation

$$[L_0] = \alpha K_1K_2 \frac{K_c}{[H^+]}[L^-]^3 - ([L_0] - 2[M_0])K_1K_2 \times \frac{K_c}{[H^+]}[L^-]^2 + \alpha K_1\left(1 + \frac{K_c}{[H^+]}\right)[L^-]^2 + \alpha[L^-] - ([L_0] - [M_0])K_1\left(1 + \frac{K_c}{[H^+]}\right)[L^-]. \quad (6)$$

The corresponding value of $[B^+]$ may then be found from

$$[B^+] = [L^-]\left(1 - \frac{[H^+]^2}{K_aK_{a'}}\right) + 2[M_0] - \frac{\alpha[L^-] - ([L_0] - [M_0])\left(2 + K_1[L^-] - K_1K_2[L^-]^2\frac{K_c}{[H^+]}\right)}{1 - K_1K_2[L^-]^2\frac{K_c}{[H^+]}}. \quad (7)$$

The amount of base required to reach any pH at which $\bar{n} = 1$ during the titration of a solution in which $[L_0] = [M_0]$ may be calculated from

$$[B^+] = \gamma'[M_0] + [OH^-], \quad (8)$$

where $$\gamma' = \frac{1 + 2K_c/[H^+] + 3K_cK_{c'}/[H^+]^2}{1 + K_c/[H^+] + K_cK_{c'}/[H^+]^2}.$$

When the titration curves thus calculated for several solutions with different values of $[L_0]/[M_0]$ are compared with those obtained experimentally it is usually found that though there is reasonable agreement on the whole, one or other of the calculated curves may show significant deviation from the observed. An appropriate modification of one or more of the constants is made until a set is obtained which provides the best fit between the expected and observed titration figures of all the solutions considered.

The values thus found for the stability and ionization constants of the complexes formed in solutions containing cupric ions and glycylglycine are $K_1 = 10^{5\cdot88}$, $K_2 = 10^{3\cdot26}$, $K_c = 10^{-4\cdot25}$, $K_{c'} = 10^{-9\cdot65}$, $K_{c''} = 10^{-10\cdot20}$. These figures were used to calculate the titration curves of solutions in which $[L_0]/[M_0] =$ 1, 2, 3 and 4. These curves are compared in Figs. 1–4, respectively, with those observed.

By the method previously described (Dobbie *et al.* 1955) copper-electrode potential measurements were made during the titration of solutions containing $CuCl_2$ and glycylglycine in the molar ratios of 1:1, 1:2 and 1:4. From these readings the corresponding values of free cupric-ion concentrations were obtained. These are compared in Figs. 1, 2 and 4, respectively, with the figures calculated from the equation

$$[M^{2+}] = \frac{[M_0]}{1 + K_1[L^-]\left(1 + \frac{K_c}{[H^+]}\right) + K_1K_2[L^-]^2\frac{K_c}{[H^+]}\left(1 + \frac{K_{c''}}{[H^+]}\right)}, \quad (9)$$

and the values of K_1, etc., given above.

As before, the copper electrode became unstable in solutions with $[Cu^{2+}]$ in the neighbourhood of 10^{-6} M or less. However, it is interesting to note, as mentioned in the previous paper, that stable

readings can be obtained in these solutions at lower cupric-ion concentrations than in solutions containing pure inorganic cupric salts.

The method of electrophoresis on filter-paper strips was used to determine the effective mean charge on the metal at various pH values in a solution containing $CuCl_2$ and glycylglycine in the molar ratio of 1:2.

The average distances traversed by samples of the solution at different pH values during 5 hr. electrophoresis with a potential gradient of 5 v/cm. are given in Table 1. These figures have been corrected for adsorption on the filter paper and are given relative to glucose which was used as a marker of the electroendosmotic flow of the glycylglycine solutions used to wet the strips. Table 1 also shows how the metal is distributed in the form of free ions and complexes at the pH values of electrophoretic examination. These figures were calculated from the values given above for the stability and ionization constants of the copper–glycylglycine complexes.

It will be seen that the pH at which the copper has no apparent electrophoretic movement is approximately the same as that at which it is expected to be present almost solely in the form of the neutral complex CuG.

Spectrophotometric observations were made at various pH values with solutions containing $CuCl_2$ and glycylglycine in the molar ratios of 1:1, 1:2, 1:3 and 1:4. Because of the greater number of complex species, the derivation of a characteristic absorption curve for each was less straightforward and more laborious than for glycine and copper.

At any pH in any of the solutions the species are present in the ratio

$$Cu^{2+}:CuG^{+}:CuG:CuG_2^{-}:CuG_2^{2-} = 1:K_1[G^{-}]:K_1[G^{-}]\frac{K_c}{[H^{+}]}:K_1K_2[G^{-}]^2\frac{K_c}{[H^{+}]}:K_1K_2[G^{-}]^2\frac{K_cK_{c'}}{[H^{+}]},$$

but generally only three of these species are simultaneously present in appreciable amounts. The absorption curve of Cu^{2+} is readily obtained and that of CuG_2^{2-} may be taken as approximately the same as the absorption curve of the 4:1 glycylglycine–Cu^{2+} solution at a high pH.

The obvious way to find the absorption curves of CuG and CuG_2^{-} would be to use the absorption curves observed for a 2:1 glycylglycine–copper solution at two pH values where only the species CuG, CuG_2^{-} and CuG_2^{2-} are present in significant amounts in solving the equations:

$$E'\alpha_{1CuG} + E''\alpha_{1CuG_2^-} + E'''\alpha_{1CuG_2^{2-}} = E_1,$$
$$E'\alpha_{2CuG} + E''\alpha_{2CuG_2^-} + E'''\alpha_{2CuG_2^{2-}} = E_2,$$

E', E'' and E''' designate the extinction coefficients for 0·005 M solutions of the complexes CuG, CuG_2^{-} and CuG_2^{2-}, respectively, at one particular wavelength; E_1 and E_2 are the experimentally observed absorption coefficients of the solution at that wavelength at the two pH values, respectively, and α_{1CuG} is the fraction of the total copper which exists in the form of the complex CuG at the pH of the first solution.

However, the range of pH in which these equations may be applied is limited, and so the coefficients α_{1CuG} and α_{2CuG} will be very similar, also the coefficients $\alpha_{1CuG_2^-}$ and $\alpha_{2CuG_2^-}$. Consequently, the

Table 1. *Electrophoretic behaviour of copper–glycylglycine complexes*

Change with pH in the electrophoretic movement of the copper in a solution containing 0·005 M-$CuCl_2$ + 0·01 M glycylglycine. Figures given are distances traversed by the copper–peptide and glucose spots on a filter-paper strip to which a potential of 5 v/cm. was applied. Corrections are made for electroendosmosis and adsorption. All distances are measured as positive in the direction of the cathode. The theoretical change with pH in charge on metal is shown by distribution of metal in form of free ions and glycylglycine complexes. G^{-} represents the glycylglycine anion.

pH	3·0	4·0	5·0	6·0	7·0	8·0	9·0	10·0
Average observed movement of copper–peptide spot (cm.)	15·3	4·9	4·5	4·5	5·5	3·1	0·3	−2·5
Average observed movement of glucose spot (cm.)	3·8	4·7	5·3	5·5	6·8	6·1	4·8	3·3
Calculated movement of copper–peptide spot due to electroendosmosis (glucose movement × R_F of copper–peptide spot)	3·2	2·0	3·9	4·8	6·2	6·1	4·8	3·3
Net movement of copper–peptide spot due to electrophoresis	12·1	2·9	0·6	−0·3	−0·7	−3·0	−4·5	−5·8
Distribution of copper (%) Cu^{2+}	98·6	72·3	7·9	0·1	—	—	—	—
Distribution of copper (%) CuG^{+}	1·4	17·7	13·9	1·7	0·1	—	—	—
Distribution of copper (%) CuG	—	10·0	77·9	94·8	77·5	44·8	29·6	23·1
Distribution of copper (%) CuG_2^{-}	—	—	0·3	3·4	22·4	54·9	66·2	47·2
Distribution of copper (%) CuG_2^{2-}	—	—	—	—	—	0·3	4·2	29·7

values calculated for E' and E'' will be very sensitive to experimental error.

Furthermore, there is the objection that this method employs only two out of all the observed absorption curves.

Perhaps the best theoretical solution would be obtained by applying the method of least squares but, in view of the great number of experimental figures, we have considered it more convenient to adopt the following method of successive approximations.

From consideration of the general changes with pH in the absorption of each solution at one wavelength and the particular value of the absorption coefficient at any pH where one species predominates it was possible to choose a likely value for the absorption coefficients of every complex at that wavelength. By inserting these figures in equations of the type shown above, absorption coefficients were calculated for solutions containing $CuCl_2$ and glycylglycine in any of the ratios used and at any of the pH values examined. Comparison of these calculated figures with those observed showed discrepancies, which suggested modifications of the values of the assumed constants, and from these modified values new calculated absorption coefficients for the solutions were obtained. If necessary, further adjustments were made until the best agreement at all pH values in all the solutions had been obtained.

When this process had been carried out at several wavelengths it became possible to deduce by graphical interpolation the approximate values of the extinction coefficients of the species at intermediate wavelengths. Further slight modifications in some of the figures were necessary to get the closest agreement between all of the calculated and observed curves.

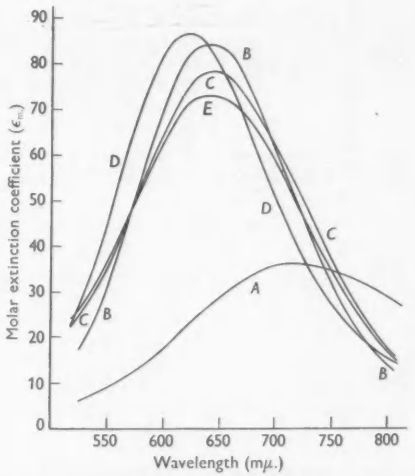

Fig. 6. Absorption curves of copper–glycylglycine complexes. Curve *A*, CuG^+; curve *B*, CuG; curve *C*, $CuG(OH)^-$; curve *D*, CuG_2^- and curve *E*, CuG_2^{2-}. (G^- represents the glycylglycine anion.)

The characteristic absorption curves thus obtained for the copper–glycylglycine complexes are given in Fig. 6. When these are combined in the proportions shown in Table 2*A* the absorption curves expected at various pH values for solutions containing different proportions of $CuCl_2$ and glycylglycine are obtained. In Table 2*B*, these calculated curves are compared with those obtained experimentally. Only a selection of the figures actually observed and calculated are given here.

It will be seen that substantial agreement is obtained between the calculated and observed values of the absorption coefficients. For any wavelength, this agreement holds over the pH range 4–11, and for a series of solutions in which the proportion of glycylglycine to copper varies from 1:1 to 4:1. This is achieved by the use of four arbitrary absorption curves, namely those of CuG^+, CuG, CuG_2^- and CuG_2^{2-}, and in addition that of $CuG(OH)^-$ which is considered only in the 1:1 solution at a pH > 8.

The fact that this wide range of agreement can be obtained with the restricted number of arbitrary constants shows that the potentiometric measurements and the spectrophotometric observations can both be accommodated quantitatively by the present theory.

Glycyl-L-leucine

The values found for the ionization constants of glycylleucine are $K_a = 10^{-8\cdot38}$ and $K_{a'} = 10^{-3\cdot12}$ at 20°.

The observed titration curves of solutions containing glycylleucine (0·005 M) and $CuCl_2$ in the molar ratios of 1:1 and 2:1 are shown in Fig. 7. These curves closely resemble those obtained with glycylglycine (Figs. 1 and 2), and complex-formation may be assumed to follow the same course.

By means of the equations derived in the section on glycylglycine, the following values were calculated from the data in Fig. 7 for the stability and ionization constants of the copper–glycylleucine complexes $K_1 = 10^{6\cdot10}$, $K_2 = 10^{3\cdot30}$, $K_c = 10^{-4\cdot80}$, $K_{c'} = 10^{-9\cdot65}$ and $K_{c''} = 10^{-10\cdot00}$.

The theoretical titration curves calculated from these constants are also shown in Fig. 7.

Glycyl-L-tyrosine

The ionization constants of the amino, carboxyl and phenolic hydroxyl groups of glycyltyrosine were found to have the values $K_a = 10^{-8\cdot37}$, $K_{a'} = 10^{-3\cdot01}$ and $K_{a_1} = 10^{-10\cdot31}$, respectively, at 20°. Greenstein (1932) reports the values $10^{-8\cdot40}$, $10^{-2\cdot98}$ and $10^{-10\cdot40}$ at 25°.

Table 2. *Copper–glycylglycine complexes*

A: distribution of copper in form of cupric ions and copper–glycylglycine complexes at various pH values in solutions containing 0·005 M-$CuCl_2, 2H_2O$ and different concentrations of glycylglycine. *B*: comparison of observed and calculated optical densities of the solutions at various pH values. The figures are log $I_0/I \times 10^3$ and have been adjusted to refer to a solution in which $[Cu_0] = 0{\cdot}005$ M at each pH. G^- represents the glycylglycine anion.

	A. Distribution of metal as				*B*. Optical densities at (mμ.)									
					550		600		650		700		750	
pH	Cu^{2+} (%)	CuG^+ (%)	CuG (%)	$CuG(OH^-)$ (%)	Obs.	Calc.	Obs.	Calc.	Obs.	Calc.	Obs.	Calc.	Obs.	Calc.
	(*a*) Glycylglycine concn., 0·005 M (composition of solution as in Fig. 1)													
3·89	88·2	8·2	3·6	—	—	—	21	23	39	41	54	55	64	65
4·89	31·9	12·7	55·4	—	98	97	207	208	252	253	195	198	121	123
7·95	—	—	98·0	2·0	170	165	355	352	417	414	300	297	157	154
8·96	—	—	83·2	16·8	175	168	349	348	410	410	302	298	162	158
10·59	—	—	10·3	89·7	181	182	326	326	391	391	306	305	178	179

	A. Distribution of metal as					*B*. Optical densities at (mμ.)									
						550		600		650		700		750	
pH	Cu^{2+} (%)	CuG^+ (%)	CuG (%)	CuG_2^- (%)	CuG_2^{2-} (%)	Obs.	Calc.	Obs.	Calc.	Obs.	Calc.	Obs.	Calc.	Obs.	Calc.
	(*b*) Glycylglycine concn., 0·01 M (composition of solution as in Fig. 2)														
4·12	64·5	20·4	15·1	—	—	—	—	75	73	103	102	105	103	94	92
5·16	4·3	10·4	84·8	0·5	—	144	146	307	311	368	369	270	273	148	150
6·90	—	0·2	80·1	19·7	—	180	178	364	364	408	410	284	286	137	149
7·83	—	—	49·3	50·5	0·2	203	199	382	383	402	404	264	271	139	143
9·00	—	—	29·6	66·2	4·2	216	211	388	390	395	398	254	262	137	140
10·43	—	—	18·6	30·2	51·2	198	196	350	351	382	381	274	276	155	155
11·00	—	—	12·0	12·0	76·0	188	189	330	331	375	372	282	282	163	162
	(*c*) Glycylglycine concn., 0·02 M (composition of solution as in Fig. 4)														
4·06	51·4	29·5	19·1	—	—	—	—	90	95	127	130	131	127	112	106
5·60	0·2	4·1	91·5	4·2	—	150	163	330	344	404	402	304	289	163	153
7·23	—	—	40·3	59·6	0·1	199	205	380	388	401	402	272	266	144	141
8·67	—	—	7·2	90·2	2·6	222	227	402	405	390	394	252	250	133	135
10·07	—	—	3·0	55·7	41·3	216	211	378	370	381	381	256	263	138	143
11·07	—	—	0·6	11·8	87·6	194	191	334	327	364	366	275	281	156	164

The observed titration curves of solutions containing $CuCl_2$ (0·005 M) and glycyltyrosine in the molar ratios of 1:1 and 1:2 are shown in Fig. 8. Inspection of the first of these curves shows that 4 equivalents of alkali/copper atom are taken up between pH 3·7 and 11·5. Here it has been assumed that complex-formation takes place as between copper and glycylglycine, with successive formation of the complexes ML^+ and ML. The latter complex then loses the ionizable proton of its phenolic OH group so that a complex ML^- is formed. Finally, in a high pH range the metal in this complex may become associated with a hydroxyl group in a complex designated $ML(OH)^{2-}$.

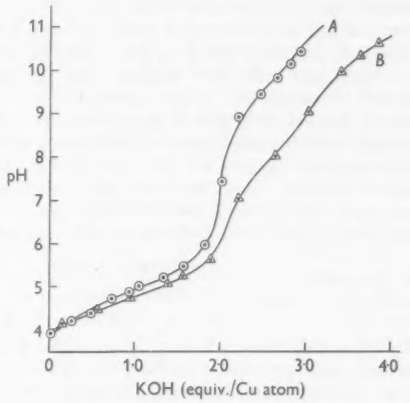

Fig. 7. Titration curves of solutions containing *A*, 23·52 mg. glycylleucine (0·005 M) + 21·31 mg. $CuCl_2,2H_2O$ (0·005 M) in 23·75 ml. *B*, 47·04 mg. glycylleucine (0·005 M) + 21·31 mg. $CuCl_2,2H_2O$ (0·0025 M) in 47·5 ml. ——, observed; ⊙ and △, calculated.

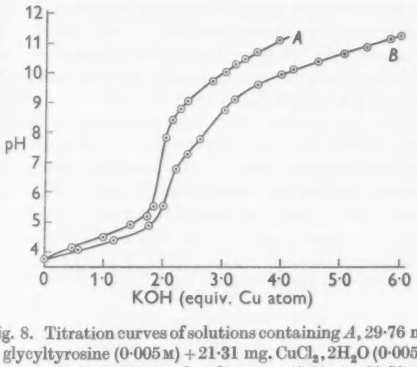

Fig. 8. Titration curves of solutions containing *A*, 29·76 mg. glycyltyrosine (0·005 M) + 21·31 mg. $CuCl_2,2H_2O$ (0·005 M) in 23 ml. *B*, 59·53 mg. glycyltyrosine (0·01 M) + 21·31 mg. $CuCl_2,2H_2O$ (0·005 M) in 21·25 ml. ——, observed; ⊙, calculated.

K_1 and K_c, the stability and peptide-bond ionization constants of ML^+, respectively, may be evaluated as for glycylglycine while

$$K_{c'} = [ML^-]\,[H^+]/[ML]$$

and

$$K_{c_1} = [ML(OH)^{2-}]\,[H^+]/[ML^-]$$

are given values consistent with the titration data above pH 7.

The validity of values thus obtained for these four constants may be checked by calculations of the theoretical titration curve which they imply for a 1:1 glycyltyrosine–copper mixture. Below $\bar{n} = 1$ this is done as for glycylglycine. When $\bar{n} = 1$ the amount of alkali required to reach any pH in this region is given by

$$[B^+] = [OH^-] + [M_0]\left(\frac{2 + 3K_{c'}/[H^+] + 4K_{c'}.K_{c_1}/[H^+]^2}{1 + K_{c'}/[H^+] + K_{c'}K_{c_1}/[H^+]^2}\right).$$

The titration curve calculated for $K_1 = 10^{5\cdot90}$, $K_c = 10^{-3\cdot85}$, $K_{c'} = 10^{-9\cdot25}$ and $K_{c_1} = 10^{-19\cdot75}$ is also shown in Fig. 8.

The titration curve of the 2:1 glycyltyrosine–copper solution shows that 6 equivalents of alkali/copper atom are taken up between pH 3·7 and 11·5. This has been interpreted as due to the occurrence of complex-formation as in a 2:1 glycylglycine–copper solution, ML_2^{2-} being formed on the addition of 4 equivalents of alkali, followed by the separate ionizations of the two phenolic hydroxyl groups in this complex to give ML_2^{3-} and ML_2^{4-}.

K_2 and $K_{c''}$, the stability and ionization constants of the complex ML_2^-, respectively, may be evaluated as for glycylglycine using the titration figures obtained during the addition of the third equivalent of alkali to the 2:1 glycyltyrosine–copper solution. Values are chosen for

$$K_{c''} = [ML_2^{3-}]\,[H^+]/[ML_2^{2-}]$$

and

$$K_{c_{IV}} = [ML_2^{4-}]\,[H^+]/[ML_2^{3-}]$$

from inspection of the titration curve above pH 9·5. When $\bar{n} < 1$, i.e. below pH 5·5, the theoretical titration curve of the 2:1 glycyltyrosine–copper solution may be calculated as for glycylglycine, putting $K_1 = 10^{5\cdot90}$ and $K_c = 10^{-3\cdot85}$. Above $\bar{n} = 1$ it may be calculated using the eqns. 10, 11 and 12:

$$\frac{(2-\bar{n})^2}{(\bar{n}-1)} = \frac{\alpha}{K_2[M_0]}\,\frac{1}{1 + \dfrac{K_{c''}}{[H^+]} + \dfrac{K_{c'}K_{c''}}{[H^+]^2} + \dfrac{K_{c'}K_{c''}K_{c_{IV}}}{[H^+]^3}}, \qquad (10)$$

where

$$\alpha = \frac{[H^+]^2}{K_aK_{a'}} + \frac{[H^+]}{K_a} + 1 + \frac{K_{a_1}}{[H^+]}$$

$$\alpha[L^-] = [M_0]\,(2-\bar{n}), \qquad (11)$$

and

$$[B^+] = (\delta' + 2)\,[M_0] + [OH^-] - \beta''[L^-], \qquad (12)$$

$$\text{where } \delta' = \frac{1+\frac{2K_{c''}}{[H^+]}+\frac{3K_{c''}K_{c'''}}{[H^+]^2}+\frac{4K_{c''}K_{c'''}K_{c_{IV}}}{[H^+]^3}}{1+\frac{K_{c''}}{[H^+]}+\frac{K_{c''}K_{c'''}}{[H^+]^2}+\frac{K_{c''}K_{c'''}K_{c_{IV}}}{[H^+]^3}},$$

and

$$\beta'' = (\delta+1)\frac{[H^+]^2}{K_aK_{a'}}+\delta\frac{[H^+]}{K_a}+(\delta-1)+(\delta-2)\frac{K_{a_1}}{[H^+]}.$$

The titration curve calculated with $K_1 = 10^{5\cdot90}$, $K_2 = 10^{3\cdot34}$, $K_c = 10^{-3\cdot85}$, $K_{c''} = 10^{-9\cdot60}$, $K_{c'''} = 10^{-10\cdot3}$ and $K_{c_{IV}} = 10^{-10\cdot7}$ is shown in Fig. 8.

Carnosine

This dipeptide is of special interest because of its biological importance and because it contains a β-alanyl group.

The values found for the ionization constants of the carboxyl, iminazole and amino groups of carnosine are $K_{a'} = 10^{-2\cdot77}$, $K_{a_1} = 10^{-6\cdot90}$ and $K_a = 10^{-9\cdot70}$ at 20°. Those reported by Deutsch & Eggleton (1938) are $10^{-2\cdot63}$, $10^{-6\cdot88}$ and $10^{-9\cdot54}$ at 22°.

The experimental titration curves of solutions containing $CuCl_2$ and carnosine in the molecular ratios of 1:1, 1:2 and 1:4 are shown in Fig. 9. Inspection of these curves reveals the following points. When $CuCl_2$ (0·005 M) and carnosine are mixed in equimolar quantities, the pH of the solution is 5·12 and on addition of 1 equivalent of alkali/copper atom the pH is raised to 5·60. Another 0·8 equivalent of alkali brings the pH to 6·50, but thereafter there is little buffering in the solution, since only a further 0·3 equivalent is required to raise the pH to 11·00. There is no evidence of buffering by the carnosine iminazole group in the neighbourhood of pH 6·9, which implies that the nitrogen atom of this group is associated with the copper atom at an early stage in the reaction

Fig. 9. Titration curves of solutions containing *A*, 28·27 mg. carnosine (0·005 M) + 21·31 mg. $CuCl_2, 2H_2O$ (0·005 M) in 23·75 ml. *B*, 56·54 mg. carnosine (0·01 M) + 21·31 mg. $CuCl_2, 2H_2O$ (0·005 M) in 22·5 ml. *C*, 113·08 mg. carnosine (0·02 M) + 21·31 mg. $CuCl_2, 2H_2O$ (0·005 M) in 22·5 ml. ——, observed; ⊙, ⊡ and △, calculated.

between metal and peptide. If the copper were thus to combine with the amino and iminazole nitrogen atoms of the carnosine molecule, then it is likely that it would also become linked to the peptide-bond nitrogen atom giving a tridentate dichelate complex from which the peptide-bond proton would be readily dissociated.

The titration curve of either of the solutions containing more than one molecule of carnosine for each cupric ion is to a first approximation the same as when the ratio is 1:1, provided allowance be made for the alkali required to titrate the ionizable groups of the extra carnosine molecules. This suggests that even when excess carnosine is present only one molecule is bound by each copper atom.

Rough calculations show that the pH of an aqueous solution containing 0·02 M carnosine and 0·005 M-$CuCl_2$, namely pH 6·64, is compatible with the suggestion that two protons are initially liberated for each 1:1 copper–peptide complex molecule formed, and that these protons combine reversibly with the free carnosine molecules present in the solution. In fact we have two protons distributed between three free carnosine molecules. These will combine with the iminazole groups of these molecules so that the pH of the solution will be

$$\text{pH} = \text{p}K_{a_1} - \log\frac{\text{combined iminazole groups}}{\text{free iminazole groups}} = 6\cdot9 - \log_{10}2 = 6\cdot6.$$

This rough analysis of the situation supports the view that one copper atom combines with the amino group of one carnosine molecule, liberating a proton and also much reducing the pK of the iminazole group, presumably as a result of bonding between the metal and the basic nitrogen atom of that group. The copper is also presumably united to the nitrogen or oxygen atom of the peptide bond and this union promotes dissociation of the proton from the amide nitrogen of that bond in the pH region 5–6. The resulting chelate structure would consist of two six-membered rings. There is apparently no tendency for a complex containing two carnosine molecules to be formed.

The only constants to be evaluated here are therefore K_1 and K_c, the stability and ionization constants respectively, of the complex ML^+. The values of these are calculated from the titration data as for glycylglycine with slight modification of eqn. 1 to take into account the extra ionization constant of carnosine itself. Eqn. 1 becomes

$$\beta''[L^-] = \gamma[L_0] - [B^+] - [H^+], \qquad (13)$$

where $\gamma = \dfrac{1+2K_c/[H^+]}{1+K_c/[H^+]}$ as before

and

$$\beta'' = \frac{(\gamma+2)[H^+]^3}{K_aK_{a_1}K_{a'}}+\frac{(\gamma+1)[H^+]^2}{K_aK_{a_1}}+\frac{\gamma[H^+]}{K_a}+\gamma-1.$$

The values found for K_1 and K_e are $10^{9\cdot65}$ and $10^{-5\cdot55}$, respectively. The theoretical titration curves calculated using these figures are also shown in Fig. 9.

DISCUSSION

In dilute aqueous solutions of simple amino acids such as glycine and divalent metal ions such as copper, the equilibrium is described by two constants K_1 and K_2, which refer to the reactions $M^2 + L^- = ML^+$ and $ML^+ + L^- = ML_2$ respectively. It now appears that when a simple dipeptide such as glycylglycine replaces the amino acid, the equilibrium can no longer be described by the two dissociation constants. However, with these dipeptides, the first reaction is still that represented by $M^{2+} + L^- = ML^+$, and so it is legitimate to compare the value of K_1 for the amino acids with the value of K_1 for polypeptides with the corresponding metal. The results of such a comparison are shown in Table 3, which confirms the conclusions of Monk (1951) and Perkins (1952) that peptide formation in general reduces the value of the first dissociation constant. However, it has to be remembered that the dissociation constants for the amino groups of the peptides are smaller than those of the acid so that at a pH of say 7·3, the relatively low stability of the complex of metal with peptide anion is to some extent compensated by the increase in the amount of anion present as compared with the amino acid under similar conditions. There must also be taken into account the effect of the equilibrium of the conversion of ML^+ into ML which promotes the tendency to complex-formation between peptide and metal. This point will be considered further in the discussion in the following paper.

The most novel result in this work as far as the dipeptides glycylglycine, glycylleucine and glycyltyrosine are concerned is the tendency of the 1 : 1 and of the 2 : 1 peptide–copper complex to react with an extra molecule of alkali in the neighbourhood of pH 4 and 10 respectively. The main problem is to decide the nature of the reaction involved, the two possibilities being (*a*) that the complex takes up a hydroxyl group and (*b*) that it gives up a hydrogen ion. As this phenomenon is not observed with simple amino acids but only with compounds containing the peptide bond, it is natural to consider that it may be dependent on some reaction between the copper atom and the —CO.NH— group. Whereas the simple uptake of a hydroxyl group by the metal could take place equally well with glycine, the loss of a hydrogen ion could only take place when a peptide bond or some similar structure was available. The conclusion that the loss of a hydrogen ion from a peptide bond is involved is borne out by the following roughly quantitative considerations.

Table 3. *Comparison of the value of K_1 for the combination of cupric ions with glycine or a dipeptide*

Chelating agent	log K_1
Glycine	8·48
Glycylglycine	5·88
Glycylleucine	6·10
Glycyltyrosine	5·90
Carnosine	8·65

The copper–glycylglycine complex CuG^+ has an apparent acid dissociation constant of value $10^{-4\cdot25}$. If this is considered in terms of hydroxyl-ion uptake it means that a complex CuG.OH is formed with dissociation constant $K_b = 10^{-10}$ approximately. Since cupric chloride (0·005 M) dissolved in water gives a solution of about pH 5·2, it would seem that the dissociation constant of CuCl.OH has the value $K_b = 10^{-6}$ approximately. This means that the complex CuG^+ has a much greater affinity for OH^- than have Cu^{2+} or $CuCl^+$. It seems, however, unlikely that the effect of chelation of the copper by the large glycylglycine anion would be to increase the affinity of the copper for hydroxyl ions. In any case an increase of the order of 10^4 would be impossible.

The neutral complex formed from CuG^+ has an absorption spectrum with $\lambda_{max.}$ at 640 mμ. and $E_{max.} = 84$. CuG^+ has $\lambda_{max.}$ at 700 mμ. with $E_{max.} = 36$. This shift in wavelength and the considerable rise in $E_{max.}$ seems to favour the interpretation of CuG as a complex containing a chelate ring rather than the complex CuG^+ with an OH^- attached.

Further evidence has been obtained from a study of the copper complexes formed by diglycylglycine. This will be discussed in a later paper, but it may be referred to here as in our opinion it strongly supports the view that the simple uptake of a hydroxyl group does not adequately explain the observed facts.

Reference may also be made to the work of Rising *et al.* (1934) and Wenaas (1937), who isolated crystalline products of the combination of copper with triglycylglycine and tetraglycylglycine, respectively. Analyses of these substances supported the view that their formation involved the loss of protons from the peptide molecules.

In the present work, no attempt was made to examine the residues obtained by evaporation of solutions containing copper and glycylglycine since these residues were extremely hygroscopic.

The observations with carnosine would seem to support the view that in complex-formation between copper and a peptide the amide link of the peptide plays an important part. As we have seen above it is difficult to avoid the conclusion that union of a cupric ion with both the nitrogen of the free NH_2 group and the basic iminazole nitrogen of carnosine takes place with great ease, giving rise to

a compound of very great stability (log $K_1 = 8{\cdot}65$). If the peptide link were not involved in the chelation system, this would imply the formation of a very stable 10-membered ring, which is unlikely. If, however, the peptide bond is associated with the metal through its nitrogen atom, then the co-ordination compound would have two six-membered rings. Such a system might well be expected to possess great stability. It has been recorded that the blue crystals of copper–carnosine possess the formula: $C_9H_{14}O_3N_4.CuO$ (Mauthner, 1913). This confirms that the stable complex is of the ML type. The actual formula is compatible with the uptake of a hydroxyl group by ML^+ or with the hydrated form of the complex minus one hydrogen ion. We have, in fact, the two situations represented by *A* and *B* in Fig. 10. If the hydrogen ion is attached to the nitrogen atom as in *A*, we have the uptake of a hydroxyl group by CuG^+ and if to the oxygen atom as in *B* then we have the uptake of a water molecule by the complex CuG.

In the light of the considerations mentioned above we tend to favour the second interpretation.

If we conclude from the above considerations that the most probable interpretation of the results is that a proton is liberated from the peptide bond as the result of chelation with the copper atom, the question arises as to whether the copper should be considered to be linked to the nitrogen or to the oxygen, i.e. are we to consider the copper as displacing the hydrogen from the peptide bond in the ketimic [—CO.NH—] form or in the enimic [—C(OH):N—] form. It appears most probable that the ionized amide group in these complexes exists as a resonance hybrid with the negative charge distributed between the oxygen and the nitrogen atoms so that the positively charged copper atom would be attracted to both. The relative importance of the two resonating forms will presumably be dependent on the precise structure of the peptides employed and it is to be expected that

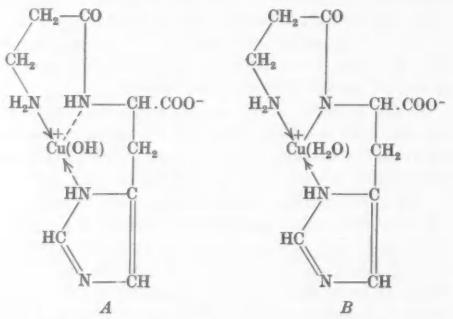

Fig. 10. Possible structural formulae for the copper–carnosine complex ML.

with carnosine for example, the nitrogen atom will be mainly involved as the linking of copper with the oxygen atom would imply the formation of a seven-membered ring.

A point of considerable interest is the very high affinity of carnosine for cupric ions and the fact that it forms only a 1:1 complex even in the presence of excess carnosine. It is one of the few dipeptides which readily gives a crystalline compound with this metal.

SUMMARY

1. Complex-formation between cupric ions and glycylglycine has been examined using the methods of potentiometric titration, spectrophotometric examination, electrophoresis and copper-electrode potential measurements. The constants characterizing the reaction between cupric ions and the dipeptide have been evaluated from the potentiometric measurements. It has been shown that the expected titration figures for solutions containing cupric chloride and glycylglycine calculated using these values of the constants agree with the experimental figures. The other experimental observations are also consistent with the theory.

2. Potentiometric measurements have been made on solutions containing cupric ions along with either glycyl-L-leucine or glycyl-L-tyrosine in various molecular proportions. Stability constants have been evaluated for the complexes formed. These are similar in magnitude to those of the copper–glycylglycine complexes. The phenolic hydroxyl group of glycyltyrosine does not appear to take part in the reaction between this peptide and cupric ions.

3. It appears that one cupric ion can combine with either one or two anions of these peptides producing complexes, each of which either loses a proton or takes up a hydroxyl group.

4. Potentiometric observations on solutions containing cupric chloride and carnosine indicate that one cupric ion combines with one carnosine molecule at three points, namely the nitrogen atom of the free NH_2 group, the basic iminazole nitrogen atom and the nitrogen or oxygen atom of the peptide bond. This produces a dichelate complex in which the co-ordinated iminazole group has no tendency to take up a proton, i.e. it has lost its salt-forming capacity. Even when carnosine is present in excess a second molecule is not apparently taken up by the metal.

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Complex-Formation between Polypeptides and Metals

3. THE REACTION BETWEEN CUPRIC IONS AND DIGLYCYLGLYCINE

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In the preceding paper (Dobbie & Kermack, 1955) a study of complex-formation between various dipeptides and cupric ions has been reported. It appeared desirable to extend this work to tripeptides, the obvious initial choice being diglycylglycine. It soon became clear that the situation was more complex than those dealt with in the preceding paper, since a greater variety of distinct molecular species were apparently formed and the potentiometric-titration data alone did not permit calculation of all the relevant constants. These were calculated from consideration of both potentiometric and spectrophotometric data. Because of the more elaborate and time-consuming calculations involved, only one tripeptide has so far been studied.

MATERIALS AND METHODS

Potentiometric titrations and spectrophotometric examinations of solutions were carried out as described previously. No copper–electrode potential measurements were made since the reliability of such readings is limited.

The diglycylglycine was obtained from Roche Products Ltd. and examined for purity on one-dimensional chromatograms run with (*a*) phenol–water (4:1, w/v), (*b*) phenol–*m*-cresol–water (2:2:1, w/v/v), or (*c*) *n*-butanol–acetic acid–water (4:1:5, by vol.) as solvent. Generally a single spot with R_F 0·55 was detected. One sample of the peptide, apparently contaminated with glycine, was recrystallized from 25% (v/v) aqueous ethanol until chromatographically pure.

RESULTS

The values found here for the ionization constants K_a and $K_{a'}$ of the amino and carboxyl group, respectively, of diglycylglycine are $10^{-8\cdot20}$ and $10^{-3\cdot24}$ at 20°. Glasstone & Hammel (1941) report the values of $10^{-8\cdot11}$ and $10^{-3\cdot19}$ at 20°.

The potentiometric-titration curves of solutions containing $CuCl_2$ (0·005 M) and diglycylglycine in the molar ratios of 1:1, 1:2, 1:4 and 1:6 are shown in Figs. 1–4, respectively. Inspection of the first of these curves reveals that three equivalents of alkali/copper atom are required to raise the pH of the solution to 10·9, one equivalent being taken up in each of the pH ranges 4·2–5·4, 5·4–6·4 and 6·4–*ca.* 8. Above about pH 8 the titration curve rises steeply. To explain these observations we assume that each cupric ion unites first with the amino nitrogen atom of a peptide anion and that the complex ML^+ thus formed reacts with two further molecules of alkali to give the complexes ML and ML^- (where L^- can represent the diglycylglycine anion).

When two molecules of diglycylglycine are present per copper atom, three equivalents of alkali/copper atom are taken up over pH ranges similar to those just mentioned and then a fourth between pH 7·5 and 11. It might appear that the process of complex-formation here is the same as in the 1:1 solution with the fourth equivalent of alkali being required to titrate the NH_3^+ group of the extra peptide molecule. As we shall see later, this interpretation does not fit in with the spectrophotometric observations made with the two solutions.

The titration curves of the solutions containing copper and diglycylglycine in the ratios of 1:4 and 1:6 correspond with the 1:2 copper–peptide curve except that two and four more equivalents of alkali/copper atom, respectively, are taken up in the neighbourhood of pH 8·2.

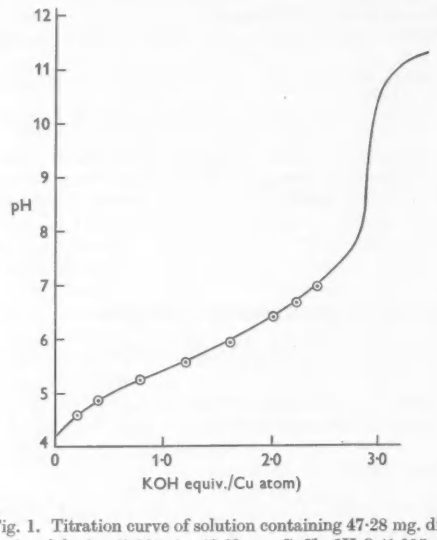

Fig. 1. Titration curve of solution containing 47·28 mg. diglycylglycine (0·005 M) + 42·63 mg. $CuCl_2, 2H_2O$ (0·005 M) in 46 ml. ——, observed; ⊙, calculated.

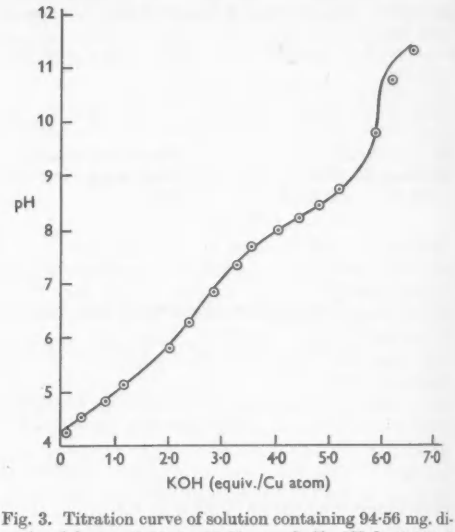

Fig. 3. Titration curve of solution containing 94·56 mg. diglycylglycine (0·02 M) + 21·31 mg. $CuCl_2, 2H_2O$ (0·005 M) in 21 ml. ——, observed; ⊙, calculated.

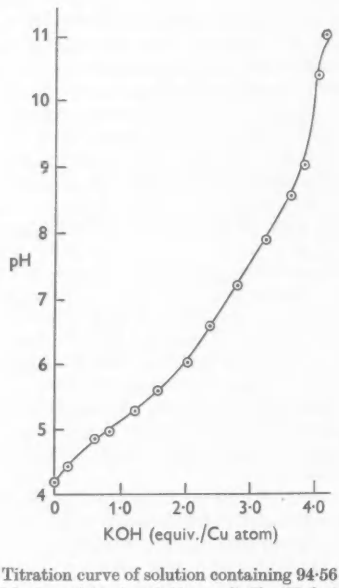

Fig. 2. Titration curve of solution containing 94·56 mg. diglycylglycine (0·01 M) + 42·63 mg. $CuCl_2, 2H_2O$ (0·005 M) in 45 ml. ——, observed; ⊙, calculated.

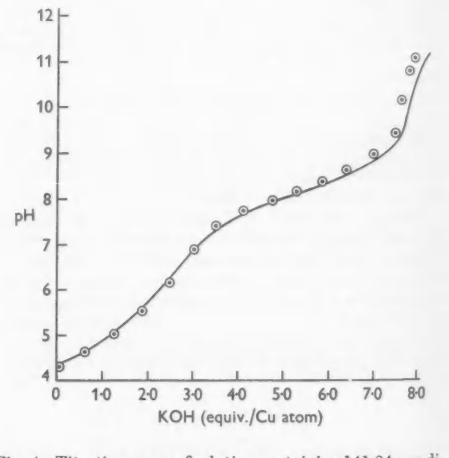

Fig. 4. Titration curve of solution containing 141·84 mg. diglycylglycine (0·03 M) + 21·31 mg. $CuCl_2, 2H_2O$ (0·005 M) in 20 ml. ——, observed; ⊙, calculated.

To account for these observations the following scheme was postulated:

$M^{2+} + L^- \rightleftharpoons ML^+$ (1) with $K_1 = \dfrac{[ML^+]}{[M^{2+}]\,[L^-]}$,

$ML^+ \rightleftharpoons ML + H^+$ (2) with $K_c = \dfrac{[ML]\,[H^+]}{[ML^+]}$,

$ML \rightleftharpoons ML^- + H^+$ (3) with $K_{c'} = \dfrac{[ML^-]\,[H^+]}{[ML]}$,

$ML + L^- \rightleftharpoons ML_2^-$ (4) with $K_2 = \dfrac{[ML_2^-]}{[ML]\,[L^-]}$,

$ML_2^- \rightleftharpoons sML_2^{2-} + H^+$ (5) with $K_{c''} = \dfrac{[sML_2^{2-}]\,[H^+]}{[ML_2^-]}$,

$ML^- + L^- \rightleftharpoons aML_2^{2-}$ (6) with $K_3 = \dfrac{[aML_2^{2-}]}{[ML^-]\,[L^-]}$,

where the prefixes s and a denote respectively the symmetrical and asymmetrical forms of the complex ML_2^{2-}. These are shown in Fig. 5. As in the case of combination between cupric ions and glycylglycine, K_1 may be calculated from the titration figures obtained during the addition of the first two equivalents of alkali (BOH) to a solution in which the initial concentrations of peptide $[L_0]$ and of metal $[M_0]$ are equal. At any pH in this range, $[L^-]$ the concentration of free peptide anions is given by

$$\beta'[L^-] = \gamma[L_0] - [B^+] - [H^+], \qquad (7)$$

where $$\gamma = \frac{1 + 2K_c/[H^+]}{1 + K_c/[H^+]}$$

and $$\beta' = (\gamma + 1)\frac{[H^+]^2}{K_a K_{a'}} + \frac{\gamma[H^+]}{K_a} + \gamma - 1,$$

and the corresponding value of $\bar{n}$ (the average number of peptide anions bound/metal ion at this pH) is given by

$$\bar{n} = 1 - \alpha[L^-]/[L_0], \qquad (8)$$

where $$\alpha = [H^+]^2/K_a K_{a'} + [H^+]/K_a + 1.$$

Hence K_1 is obtained from

$$K_1 = \frac{\bar{n}}{(1-\bar{n})\,[L^-]}\,\frac{1}{(1 + K_c/[H^+])}. \qquad (9)$$

Values of K_c, consistent with the titration data, were tried in eqns. 7, 8 and 9 until that value was found which provided the most constant set of values for K_1 when calculated at many points on this part of the titration curve. The values thus found are $K_1 = 10^{5\cdot30}$ and $K_c = 10^{-5\cdot20}$.

From consideration of the titration figures obtained during the addition of the third equivalent of alkali to a solution in which $[L_0] = [M_0]$, $K_{c'}$ was given the value of $10^{-7\cdot0}$.

Some difficulty was encountered when a procedure similar to that used for K_2 with glycylglycine was adopted to evaluate the stability constants K_2 and K_3 in the diglycylglycine–copper system. This difficulty arose from the fact that the value of $[L^-]$ above pH 6 in a solution where $[L_0]/[M_0]\ (= X) > 1$ cannot be calculated without making use of either K_2 or K_3. $[L^-]$ may be found from either

$$\left(\frac{\alpha\epsilon K_3}{1 + K_{c''}/[H^+]}\right)[L^-]^2 - \left(\frac{\epsilon K_3[M_0]\,(X-2)}{1 + K_{c''}/[H^+]} + \beta''\right)[L^-] + (2 + \epsilon_1(X-2) + \delta(X-1))\,[M_0] = [B^+], \qquad (10)$$

where $$\epsilon = \frac{K_c K_{c'}/[H^+]^2}{1 + K_c/[H^+] + K_c K_{c'}/[H^+]^2}$$

$$\epsilon_1 = \frac{1 - K_c K_{c'}/[H^+]^2}{1 + K_c/[H^+] + K_c K_{c'}/[H^+]^2}$$

$$\delta = \frac{1 + 2K_{c''}/[H^+]}{1 + K_{c''}/[H^+]}$$

and

$$\beta'' = (\delta + \epsilon_1 + 1)\frac{[H^+]^2}{K_a K_{a'}} + (\delta + \epsilon_1)\frac{[H^+]}{K_a} + \delta + \epsilon_1 - 1$$

or a similar equation in K_2, and thereafter K_2 or K_3 may be found from

$$K_2 = \frac{[M_0]\,(X-1) - \alpha[L^-]}{(\alpha[L^-] - [M_0]\,(X-2))\,\epsilon_{11}[L^-]} - \left(\frac{\dfrac{K_{c'}}{[H^+]}}{1 + \dfrac{K_{c''}}{[H^+]}}\right)K_3, \qquad (11)$$

where $$\epsilon_{11} = \frac{\left(1 + \dfrac{K_{c''}}{[H^+]}\right)\dfrac{K_c}{[H^+]}}{1 + \dfrac{K_c}{[H^+]} + \dfrac{K_c K_{c'}}{[H^+]^2}}.$$

It was found to be impossible to decide upon the most likely values of K_2, K_3 and $K_{c''}$ by using these equations and the usual method of successive approximations.

So it was decided to estimate K_2 from the titration data for a 2:1 diglycylglycine–copper solution in the pH range 6–7, where comparatively little of the species aML_2^{2-} would be expected to be present, putting $K_3 = 0$ in eqns. 10 and 11 and giving $K_{c''}$ various values in the neighbourhood of 10^{-8}. For values of $K_{c''}$ about $10^{-8\cdot5}$ a relatively constant value of $10^{3\cdot7}$ was thus obtained for K_2 in the pH range 6–7. With these values for K_2 and $K_{c''}$, eqns. 10 and 11 were applied to the titration data obtained above pH 7·5 for the 2:1 and 4:1 diglycylglycine–copper mixtures. The values thus found for K_3 were of the order 10^2.

In order to establish the accuracy of these values calculated for K_2, K_3 and $K_{c''}$ it was necessary to consider also the spectrophotometric observations made on the copper–peptide solutions. During the addition of a third equivalent of alkali to a solution containing equimolar quantities of diglycylglycine

Table 1. *Copper–diglycylglycine complexes*

A: distribution of copper in form of cupric ions and copper–diglycylglycine complexes at various pH values in solutions containing 0·005 M-$CuCl_2,2H_2O$ and different concentrations of diglycylglycine. *B*: comparison of observed and calculated optical densities of the solutions at various pH values. The figures are log $I_0/I \times 10^3$ and have been adjusted to refer to a solution in which $[Cu_0] = 0{\cdot}005$ M at each pH. G^- represents the diglycylglycine anion.

	A. Distribution of metal as					*B*. Optical densities at (mμ.)									
						550		600		650		700		750	
pH	Cu^{2+} (%)	CuG^+ (%)	CuG (%)	CuG^- (%)	CuG_2^- (%)	Obs.	Calc.	Obs.	Calc.	Obs.	Calc.	Obs.	Calc.	Obs.	Calc.
	(*a*) Diglycylglycine concn., 0·005 M (composition of solution as in Fig. 1)														
5·06	58·0	24·2	17·5	0·2	0·1	—	—	59	60	102	100	113	116	107	110
5·93	15·1	12·3	65·9	5·6	1·1	118	113	218	213	288	282	257	255	174	176
6·95	1·3	0·9	51·3	45·8	0·7	415	426	413	417	328	331	224	223	131	127

	A. Distribution of metal as						*B*. Optical densities at (mμ.)											
							500		550		600		650		700		750	
pH	Cu^{2+} (%)	CuG^+ (%)	CuG (%)	CuG^- (%)	CuG_2^- (%)	CuG_2^{2-} (%)	Obs.	Calc.	Obs.	Calc.	Obs.	Calc.	Obs.	Calc.	Obs.	Calc.	Obs.	Calc.
	(*b*) Diglycylglycine concn., 0·01 M (composition of solution as in Fig. 2)																	
4·83	52·5	33·1	14·1	0·1	0·2	—	—	—	—	—	—	—	94	95	115	116	114	115
5·78	1·9	17·9	68·1	4·1	5·4	—	—	—	115	111	227	225	306	303	276	275	186	190
6·86	—	0·9	41·7	30·2	26·3	0·9	171	167	342	338	380	376	327	329	228	228	133	132
7·88	—	—	7·1	53·9	28·3	10·7	294	294	527	529	463	464	280	288	143	143	59	59
10·36	—	—	—	61·7	0·3	38·0	272	273	616	617	476	475	236	234	86	87	—	—
	(*c*) Diglycylglycine concn., 0·02 M (composition of solution as in Fig. 3)																	
4·53	53·2	38·5	8·2	0·1	—	—	—	—	—	—	—	—	83	78	114	104	119	111
5·82	1·3	15·4	64·1	4·2	15·0	—	—	—	124	125	236	243	306	315	271	276	184	187
6·82	—	0·6	27·0	17·9	52·7	1·8	116	120	264	273	337	345	313	320	218	220	128	129
7·97	—	—	2·8	26·5	48·2	22·5	194	198	369	373	371	372	264	268	148	149	79	77
9·22	—	—	0·2	29·9	7·5	62·4	276	277	445	445	360	358	193	196	83	86	—	—
11·31	—	—	—	30·4	0·1	69·5	286	290	454	456	361	356	186	184	73	76	—	—
	(*d*) Diglycylglycine concn., 0·03 M (composition of solution as in Fig. 4)																	
4·65	34·9	50·4	14·2	0·1	0·4	—	—	—	—	—	62	64	112	111	144	137	143	136
5·53	2·5	26·7	57·0	1·9	11·9	—	—	—	92	94	194	207	268	283	253	261	182	187
6·87	—	0·4	17·5	13·0	66·6	2·5	103	104	246	251	329	334	308	314	215	211	126	123
8·18	—	—	1·1	17·3	46·4	35·2	179	180	327	331	334	336	243	246	139	139	75	73
9·44	—	—	—	19·0	5·4	75·6	255	252	390	391	315	316	170	175	79	79	—	—
11·28	—	—	—	19·3	0·1	80·6	261	261	397	399	316	313	166	166	71	72	—	—

and $CuCl_2$, the colour of the solution changes from deep blue through violet to purple. The actual change in absorption spectrum is shown in Table 1*B*. It will be seen that in the neighbourhood of pH 7 the solution has a high absorption peak at about 550 mμ. This absorption is quite different in position and intensity from any we have observed with dipeptides or amino acids, an observation which agrees with the view that here a new type of complex, namely a tridentate dichelate structure is present in considerable concentration. We have thus ascribed an absorption spectrum with $\lambda_{max.}$ in the neighbourhood of 550 mμ. to complexes with this structure, i.e. to ML^- and aML_2^{2-}.

From Table 1*B* we see also that the 2:1, 4:1 and 6:1 peptide–copper solutions in the pH range 7–10 have each an absorption maximum in the neighbourhood of 550–560 mμ. However, with increase in peptide concentration the intensity of absorption at this maximum is lowered. This implies that in the presence of a large concentration of tripeptide anions the absorption of the species ML^- is affected, and this can only arise if these anions attach themselves to the complex. It is for this reason that we associate the uptake of the fourth equivalent of alkali/copper atom in the solution containing peptide and copper in molar ratio of 2:1 with the formation of aML_2^{2-} shown in Fig. 5. To account for the change in intensity of absorption, the complex aML_2^{2-} must have an absorption spectrum with $\epsilon_{max.}$ much less than that of ML^-. Since sML_2^{2-} and aML_2^{2-} are isomers which exist in any solution in an equilibrium unaffected by pH or proportion of peptide to metal, their individual absorption spectra cannot be found from the data given here. Instead an absorption curve has been assigned to ML_2^{2-} which denotes the two complexes taken together in their equilibrium ratio

$$sML_2^{2-}/aML_2^{2-} = K_{c''}.K_2/K_{c'}.K_3.$$

In order to enable definite arithmetical calculations to be made it is necessary to give this fraction a value. The value taken here is unity, although the actual value might be substantially less than this since the wavelength of maximum absorption of a solution in which these complexes are present in considerable concentration is about 550 mμ. By analogy with ML^- we expect aML_2^{2-} to have $\lambda_{max.}$ in this neighbourhood, while sML_2^{2-} might be expected to have an absorption spectrum similar to that of the corresponding complex of glycylglycine, i.e. with $\lambda_{max.}$ about 620 mμ. If the value is less than 1, then an appropriate modification would have to be made in the values given here for K_3 and also $K_{c''}$ in order to satisfy both the potentiometric and spectrophotometric data.

Putting $K_{c'} = 10^{-7\cdot0}$, $K_{c''} = 10^{-8\cdot4}$, $K_2 = 10^{3\cdot7}$ and making the above fraction = 1, we obtain $K_3 = 10^{2\cdot3}$. Using these values along with $K_1 = 10^{5\cdot3}$ and $K_c = 10^{-5\cdot2}$, tentative absorption curves for the

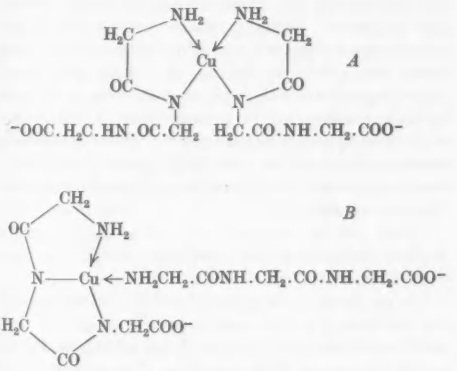

Fig. 5. Proposed structural formulae of *A*, the symmetrical copper–diglycylglycine complex $sCuG_2^{2-}$ and *B*, the asymmetrical copper–diglycylglycine complex $aCuG_2^{2-}$. (G^- represents the diglycylglycine anion.)

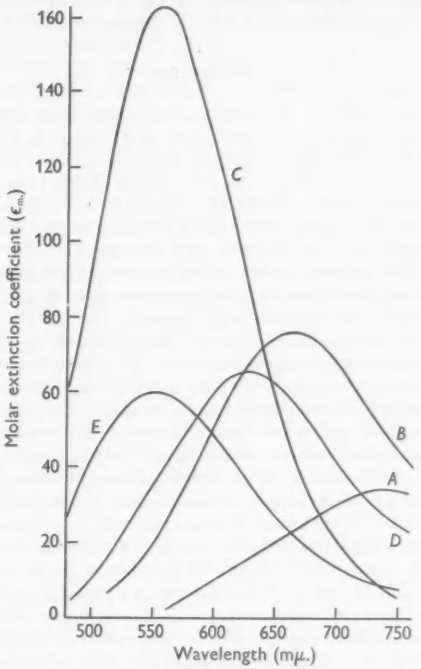

Fig. 6. Computed absorption curves of copper–diglycylglycine complexes. Curve *A*, CuG^+; curve *B*, CuG; curve *C*, CuG^-; curve *D*, CuG_2^- and curve *E*, CuG_2^{2-}. (G^- represents the diglycylglycine anion.)

various molecular species were derived as described for glycylglycine. However, when the composite absorption curves were recalculated and compared with the observed values, although there was broad general agreement there were a number of divergencies. This suggested that the values of the constants required some adjustment. Suitable modifications were therefore made on an empirical basis taking care that the theoretical titration curves of all four solutions calculated using these modified constants in eqns. 7–11 remained consistent with those observed.

It was found that the best agreement between all of the observed and calculated figures was obtained when the following values were given to the reaction constants: $K_1 = 10^{5\cdot3}$, $K_2 = 10^{3\cdot6}$, $K_3 = 10^{2\cdot0}$, $K_c = 10^{-5\cdot2}$, $K_{c'} = 10^{-7\cdot0}$ and $K_{c''} = 10^{-8\cdot6}$.

The potentiometric-titration curves calculated using these figures are shown in Figs. 1–4, and the absorption curves derived for the copper–diglycylglycine complexes in Fig. 6. A selection of the spectrophotometric figures calculated by combination of the latter set of curves with the figures showing the distribution of the copper among the various complexes formed at any pH (Table 1*A*) are compared in Table 1*B* with those observed.

DISCUSSION

If we compare the experimental observations for copper and glycylglycine with those reported here we find that there are three main differences. The first comes to light on considering the potentiometric-titration curves of solutions containing one molecule of glycylglycine or diglycylglycine for each cupric ion. In the first case two equivalents of KOH/copper atom are taken up over the pH range 4–8, whereas with diglycylglycine three equivalents of KOH are required in this range.

Secondly, during the addition of alkali to the glycylglycine–copper solution the colour of the solution deepens from pale greenish blue to very dark blue. With diglycylglycine a similar deepening of colour is observed during the addition of the first two equivalents of alkali/copper atom, but thereafter the colour of the solution changes through violet to deep purple, while the third equivalent of alkali is being added. This corresponds to an actual shift in the wavelength of maximum absorption of the solution from about 650 mμ. to about 550 mμ.

Thirdly, on considering solutions containing two or more molecules of diglycylglycine/copper atom, we find that the intensity of absorption in the pH range 9–11 decreases substantially with increase in peptide concentration, whereas with glycylglycine only a very slight falling off in intensity is observed.

These three distinctions between the behaviour of glycylglycine and diglycylglycine in the presence of cupric ions can be explained by the existence, in the case of the tripeptide, of a dichelate complex ML^-. The formation of such a complex was postulated initially to account for the uptake between pH 6·5 and 8·5 of a third equivalent of alkali by the copper in a solution containing equimolar quantities of $CuCl_2$ and diglycylglycine. To this complex we may assign a characteristic absorption spectrum with maximum absorption at a much lower wavelength than that of a simple monochelate complex. In the presence of excess peptide the concentration of ML^- is reduced as it becomes replaced by a 2:1 peptide–metal complex with much lower absorption.

These results have a bearing on the question as to how far the uptake of alkali during the potentiometric titrations of copper–peptide mixtures is to be attributed to the direct attachment of OH groups to the metal or to the release of protons from the peptide. If we consider complexes of the type ML formed by glycylglycine and by diglycylglycine, there would appear to be no reason why the first should not take up a hydroxyl group as easily as the second. On the other hand, the first could not lose a proton, as it has no free amide link, while the second might be expected to lose a proton with considerable ease. The fact that the 1:1 diglycylglycine–copper solution readily takes up almost three equivalents of alkali below pH 8, while a similar glycylglycine solution only takes up a third equivalent of alkali in the pH range 9–11 after a very steep rise in the titration curve suggests that the two complexes of type ML^- thus formed after the addition of three equivalents of alkali are quite different in structure. In the case of glycylglycine, it is difficult to assign to this complex a structure other than $ML.OH^-$, so that for the diglycylglycine complex to be different it must be formed by the loss of a proton from the second amide link of this peptide. Actually there was some slight experimental suggestion that a fourth equivalent of alkali was taken up by the 1:1 diglycylglycine–copper solution in a high pH region. However, some turbidity developed in the solution at this point with consequent disturbance of the equilibrium between the various molecular species, so that it was not possible to obtain reliable potentiometric-titration readings.

These results support the view that copper–peptide complexes may lose the protons of their peptide links.

We may refer here to the possibility mentioned in the preceding paper that a proton might be lost not from a peptide bond but from a hydrated form of the copper–peptide complex. This is equivalent to the uptake of a hydroxyl group by the copper atom. However, in the tridentate copper complex which we now postulate with diglycylglycine a difficulty would arise in this interpretation. We

would have attached to one copper atom three nitrogen atoms and two oxygen atoms, these latter being the oxygens of the two water molecules. Such a complex containing 5-co-ordinated copper would seem to be unlikely. We are therefore inclined to favour the view that the uptake of alkali is the result of the direct loss of protons from the amide groups of the copper–peptide complexes.

We have already referred to the fact that the uptake of the fourth equivalent of alkali by the solution containing two molecules of peptide/copper atom takes place at a pH value which corresponds roughly to the pK_a of the $—NH_3^+$ group of the peptide. This means that only when considerable concentration of chelating anion, i.e. the peptide anion L^-, has been produced does any appreciable amount of complex-formation take place. In accordance with this the value of K_3 is small, namely 10^2, which may be compared with $K_1 = 10^8$. When complex formation takes place with a K of the latter magnitude or greater, there is considerable complex-formation at pH values much below pK_a, that is in the presence of even very low concentration of the chelating anion L^-. The consequent reduction in the concentration of L^- results in further dissociation of the $—NH_3^+$ groups of the zwitterion. Thus, though we tend to regard the liberation of protons as due to the combination of the zwitterion with the metal, yet the immediate source of the protons is the dissociation of the $—NH_3^+$ groups of the zwitterions into NH_2 and H^+. When the value of K is smaller, there is no essential change in the process of complex-formation and proton-liberation, but as remarked above, little complex-formation now takes place until dissociation of the $—NH_3^+$ groups is considerable. The uptake of the resulting anions by the metal still promotes further dissociation, but the importance of this effect is now relatively slight, as the pH is so high that dissociation of the zwitterion into proton and anion is already considerable. Thus it is not altogether incorrect to say that in the potentiometric titration the uptake of the fourth equivalent of alkali is due to the pK_a of the peptide, but the equilibrium is modified somewhat by the simultaneous association of the anion with the metal. This description of the process remains true even when the value of K is high and the equivalent of alkali is taken up at a much lower pH, only here the modifying effect of chelation on the equilibrium becomes predominant.

The situation with regard to the uptake of the third equivalent of alkali is especially interesting from this point of view. Here we are concerned with the conversion of ML into ML_2^- with $K_2 = 10^{2\cdot6}$, the third proton being given up in the neighbourhood of pH 6–7. This value of K_2 is sufficiently high to displace the uptake substantially below the pH range corresponding to the pK_a of the $—NH_3^+$ group. Of course the situation is further complicated by the other equilibria in which ML and ML_2^- are involved, but the main effect is that indicated above.

It is of interest to compare the ability of diglycylglycine and the peptides previously studied to bind cupric ions at the physiologically interesting pH of 7·3 since this gives some indication of how these substances would compete with each other for free cupric ions.

In Table 2 is shown the distribution of the metal in the form of free ions and complexes in each of a series of solutions containing cupric chloride (0·005 M) and either glycine or one of the peptides studied (0·005 M).

It appears that under these conditions of concentration and pH the order of cupric-ion binding by these reagents is

glycyltyrosine > carnosine > glycylglycine > glycylleucine > diglycylglycine > glycine.

Though the value of the stability constant K_1 is higher for glycine ($10^{8\cdot48}$) than for any of these peptides except carnosine, the uptake of cupric ions by any one of the peptides under these conditions would be greater than that by glycine. This is partly to be attributed to the fact that the value of pK_a for any one of these peptides (except carnosine) is significantly lower than for the simple amino acid so that at pH 7·3 a greater proportion of each peptide exists as the free chelating anion. There is the further factor that the conversion of the copper–peptide complex ML^+ into ML by the intramolecular loss of a proton results in a shift of equilibrium which causes more copper to be taken up by the

Table 2. *Distribution of copper in form of free ions and complexes in solutions each containing* $CuCl_2$ (0·005 M) *and either glycine or a peptide* (0·005 M) *at* pH 7·3

Chelating agent	Free ligand concn. $[L^-]$	Distribution of metal (%)						
		Cu^{2+}	CuL^+	CuL	CuL^-	CuL_2	CuL_2^-	CuL_2^{2-}
Glycine	$10^{-7\cdot74}$	13·3	73·4	—	—	13·3	—	—
Glycylglycine	$10^{-6\cdot29}$	0·23	0·09	99·15	0·44	—	0·09	—
Glycylleucine	$10^{-6\cdot13}$	0·34	0·31	98·76	0·44	—	0·15	—
Glycyltyrosine	$10^{-6\cdot52}$	0·15	0·03	98·64	1·11	—	0·07	—
Carosine	$10^{-7\cdot63}$	0·17	1·73	98·10	—	—	—	—
Diglycylglycine	$10^{-5\cdot69}$	0·64	0·26	32·94	65·87	—	0·26	0·03

Table 3. *Free cupric-ion concentration in solutions each containing* $CuCl_2$ (0·005 M) *and either glycine or a peptide* (0·025 M) *at* pH 7·3

Chelating agent	Free cupric-ion concentration (M)
Glycine	$10^{-9.01}$
Glycylglycine	$10^{-8.86}$
Glycylleucine	$10^{-8.56}$
Glycyltyrosine	$10^{-9.33}$
Carnosine	$10^{-8.46}$
Diglycylglycine	$10^{-8.96}$

peptide. It is true that the copper–glycine complex of the type ML^+ readily takes up a second glycine anion. However, under the conditions envisaged here, where the copper:ligand ratio is 1:1, the extent to which this reaction can take place is limited and therefore its effect on copper binding is less marked than that of proton loss by the copper–peptide complex ML^+.

On inspection of Table 3, which shows the free cupric-ion concentration at pH 7·3 in a series of solutions each containing cupric chloride (0·005 M) and one of the chelating agents in the molar ratio of 1:5 we find that the order of metal binding under these conditions would be

glycyltyrosine > glycine > diglycylglycine > glycylglycine > glycylleucine > carnosine.

In these solutions containing excess chelating agent the order of copper binding is now markedly influenced by the value of K_2. Since the value of K_2 for glycine ($10^{7.6}$) is very much higher than for any of the peptides, the amino acid is now second in the list, glycyltyrosine with its low pK_c still remaining at the top. Furthermore, the inability of the copper–carnosine complex ML to take up a second carnosine anion results in this peptide being displaced to the foot of the list.

It is clear that the competing powers of various ligands for copper depends to a considerable extent on the ratio of copper atoms to ligand molecules.

SUMMARY

1. Potentiometric titrations and spectrophotometric observations have been made with solutions containing cupric chloride (0·005 M) and diglycylglycine in the ratio of 1:1, 1:2, 1:4 and 1:6.

2. The observations have been interpreted on the assumption of the existence in these solutions of the copper–diglycylglycine complexes ML^+, ML, ML^-, ML_2^-, symmetrical ML_2^{2-} and asymmetrical ML_2^{2-}. Values have been calculated for the stability and dissociation constants of these complexes, and it is shown that these values are consistent with the experimental observations. A characteristic absorption spectrum has been assigned to each of these complexes.

3. The results support the view that in the process of formation of such complexes as ML, a link is established between the amide group and the copper atom, with the loss of a hydrogen ion.

4. The relative avidities of various glycine-containing peptides for cupric ions are compared with each other and with that of glycine itself, at the approximate physiological pH of 7·3.

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The Incorporation of ^{65}Zn in Mammary Tumours and Some Other Tissues of Mice after Injection of the Isotope

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The zinc content of normal tissues has been widely studied (Rost & Weitzel, 1919; Delezenne, 1919; Rost, 1921; Lutz, 1926; Dutoit & Zbinden, 1930; Simakov, 1940; Eggleton, 1940; Gettler & Bastian, 1947). Pathological material has been studied less extensively, and the zinc content of neoplastic tissue has been compared with that of normal tissues by only a few groups of workers. Some investigators (Cristol, 1923; Labbé & Nepveaux, 1927; Sugai, 1939, 1940; Addink, 1950) found that the concentration of zinc in some tumours was higher than that in normal tissues. On the other hand, Carruthers & Suntzeff (1945, 1946) and Cowdry, Carruthers & Suntzeff (1948) observed a decrease in the zinc content of epidermis associated with methylcholanthrene-induced tumours in this

tissue, and leukocytes from leukaemic subjects have been found to contain less zinc than normal leukocytes (Gibson, Vallee, Fluharty & Nelson, 1950).

In view of these conflicting observations on the zinc content of tumour tissues in relation to that of normal tissues, we decided to use ^{65}Zn to study the relative uptakes of zinc by spontaneously occurring tumours and the corresponding non-malignant tissue following a single intraperitoneal injection of the isotope. The tumour selected for the experiments described in this paper was the mammary gland tumour which develops in a high proportion of mice of certain pure-line strains. The 'control' tissues were the apparently non-malignant portions of the mammary glands of these injected tumour mice and also the mammary glands of normal non-tumour-bearing mice which had been injected with ^{65}Zn at the same time as were the tumour mice.

The main object of these investigations was to determine whether malignant tissue differs from the corresponding normal tissue in its uptake or utilization of zinc, and these experiments form part of a study of the feasibility of obtaining specific deposition of radioactive isotopes in malignant tissue. In addition to the mammary glands and tumours a few other tissues of the injected mice were examined for ^{65}Zn in these experiments, to determine whether the tumour mice differ from normal mice in their utilization of injected zinc.

EXPERIMENTAL

Mice

Pure-line female mice of the 'C3H', 'Strong-A', and 'ABC' strains, bred in our laboratory from strains supplied to us by the Institute of Cancer Research (Royal Cancer Hospital) and the Imperial Cancer Research Fund Laboratories, were used for the experiments with tumour-bearing animals. Almost all the females of these strains ultimately develop mammary carcinomata spontaneously. Female mice of a mixed strain bred in our laboratory and showing a very low incidence of spontaneously occurring mammary carcinomata were used as controls.

All the tumour mice and most of the control animals had had one or more litters of young before being used for experimental purposes. If for any reason (e.g. cancer development in the mother) it was not possible for the young pure-line mice to be suckled by their own mothers they were foster-mothered by a lactating female of the same strain.

The animals were housed in galvanized-iron box cages and were fed on food pellets (diet 41, Associated London Flour Millers), oats and water, with a supplement of lard, milk and crushed dog biscuits on 1 or 2 days in each week.

Preparation of ^{65}Zn for injection

Radioactive zinc of high specific activity was separated as $ZnCl_2$ from cyclotron-irradiated copper by one of the procedures previously described (Tupper & Watts, 1954; Tupper, Watts & Wormall, 1954). As in previous animal experiments, we have found it convenient to inject the zinc as a zinc–glycine complex, for in this form the zinc is held in solution at pH 7·4 and yet it is readily available for biological utilization. This complex was prepared by dissolving 10 mg. glycine in 1 ml. of the ^{65}Zn-containing solution and adding N-NaOH to give a pH of 7·4 and water to give a final volume of 5 ml. The final solution contained 6 μg. Zn/ml. and gave counting rates of $8{\cdot}25 \times 10^4$ and $1{\cdot}9 \times 10^6$ counts/min./ml. with the Veall-type (M. 6) and scintillation counter, respectively.

Injection of ^{65}Zn

The injections were made intraperitoneally, and each mouse received 0·025 ml. of the ^{65}Zn-containing solution/g. body weight.

The sixty tumour-bearing mice used in these experiments received their injections a few days after mammary tumours had appeared, and groups of these mice (8–10 in each group) were killed 3 hr., 1, 3, 7 and 14 days after the injection. A similar series of experiments was carried out with sixty control (non-tumour-bearing) mice. In the planning of the experiment it was intended that one group of injected mice would be killed 21 days after the injection but several of the tumour-bearing mice in this group were killed and discarded at an earlier stage since ulceration of the tumour through the skin appeared imminent. Thus results were obtained with only four tumour-bearing mice in this group (group 6), and four control mice, and in view of this small number of observations these results are not included in the Tables of this paper.

Removal of tissues

The mouse was killed with coal-gas, and a blood sample was immediately taken from the heart. No attempt was made to render the organs blood-free by perfusion.

Mammary tissue was removed by blunt-dissection, and care was taken to remove all visible lymph-nodes from the portions used for analysis. The identity of the normal mammary tissue was checked histologically in the case of the first thirty normal (non-tumour-bearing) mice used, and this precaution was only abandoned when it had been established that the technique employed ensured that mammary gland tissue containing no lymphoid tissue could be consistently obtained. A similar histological check was made on the 'normal' mammary gland tissues of the tumour-bearing mice. Between 150 and 500 mg. wet weight mammary tissue were obtained from each mouse.

The first nineteen mammary tumours investigated, and any in unusual situations or with unusual macroscopic appearances, were examined histologically and found to be typical anaplastic carcinomata.

Determinations

Radioactive zinc (^{65}Zn). For most of these determinations the tissue sample was digested with H_2SO_4 and the radioactivity of the digest measured with a scintillation counter (as described by Banks, Tupper, Watts & Wormall, 1954*a*, *b*), but a few of the earlier determinations were made by the older procedure of Banks, Tupper & Wormall (1950) using a Veall (1948)-type liquid counter. Measurements were made for sufficient time to give a total count with a probable error of $\pm 1\%$ when the scintillation-counter was used and $\pm 2{\cdot}5\%$ when Geiger–Müller counters were employed.

Total nitrogen. Total N was determined by the micro-Kjeldahl method, the ammonia being distilled in the apparatus described by Markham (1942), into a boric acid solution containing methyl red–methylene blue indicator.

RESULTS

It was not possible to separate the ducts and alveoli, which are the mammary gland components from which carcinomata arise, from the relatively large amounts of fat in which they are embedded. Because of this variable fat content of the samples it was considered that ^{65}Zn contents based on a unit weight of tissue would have little significance. We have, therefore, related the amount of ^{65}Zn in each tissue to the nitrogen content, and the results are expressed as the percentage of the injected ^{65}Zn/mg. tissue nitrogen.

As pointed out by Thomson, Heagy, Hutchison & Davidson (1953), the relating of the results of tissue analysis to dry or wet weight of the tissue has serious disadvantages, and they advocate the expression of analytical results in relation to deoxyribonucleic acid content. Unfortunately this method could not be adopted in our present investigations, since the amount of tissue available was often too small for the necessary determinations.

Comparison of mammary gland carcinoma with 'normal' (apparently non-malignant) mammary gland tissue

The results presented in Table 1 show that, except with the mice killed 3 hr. after the injection of ^{65}Zn (i.e. group 1) the carcinoma tissue had a higher mean ^{65}Zn/total N ratio than had the 'normal' mammary gland tissue from either

Table 1. *Comparison of the* ^{65}Zn *contents of mammary gland carcinoma and normal mammary gland tissue*

The mice were given a single intraperitoneal injection of ^{65}Zn-containing zinc–glycine. The ^{65}Zn contents of the tissues are given as mean values ± standard error. The number of tumours or mammary glands examined (the whole mammary gland tissue obtained from each mouse being counted as one gland) are given in brackets.

Group	Interval between injection and killing (days)	^{65}Zn contents (as % injected ^{65}Zn/mg. tissue N)		
		Mammary gland		Mammary gland carcinoma
		Non-tumour-bearing mice	Tumour-bearing mice*	
1	0·125	0·164 ± 0·046 (8)	0·143 ± 0·031 (8)	0·134 ± 0·038 (8)
2	1	0·162 ± 0·019 (6)	0·128 ± 0·017 (7)	0·248 ± 0·046 (9)
3	3	0·153 ± 0·034 (7)	0·102 ± 0·006 (5)	0·235 ± 0·015 (12)
4	7	0·096 ± 0·019 (7)	0·075 ± 0·013 (7)	0·148 ± 0·011 (9)
5	14	0·052 ± 0·008 (9)	0·043 ± 0·006 (7)	0·065 ± 0·007 (10)

* The 'normal' mammary gland tissues from tumour-bearing mice were obtained from the same mice as the corresponding carcinomata, but in a few cases, particularly with mice, with large tumours, it was not possible to obtain sufficient of the 'normal' tissue for analysis.

Table 2. *The* ^{65}Zn *contents of some tissues of tumour-bearing and non-tumour-bearing mice following an intraperitoneal injection of* ^{65}Zn*-containing zinc–glycine*

Group	Interval between injection and killing (days)	^{65}Zn content (as % injected ^{65}Zn/mg. tissue N)									
		Liver		Lung		Kidney		Skin		Blood	
		T*	NT*	T	NT	T	NT	T	NT	T	NT
1	0·125	0·527 ± 0·107 (8)	0·427 ± 0·084 (7)	0·137 ± 0·011 (8)	0·153 ± 0·020 (8)	0·348 ± 0·038 (8)	0·289 ± 0·019 (7)	0·055 ± 0·015 (8)	0·065 ± 0·015 (8)	0·095 ± 0·025 (8)	0·154 ± 0·056 (7)
2	1	0·348 ± 0·032 (9)	0·401 ± 0·030 (8)	0·154 ± 0·013 (9)	0·181 ± 0·023 (6)	0·217 ± 0·016 (9)	0·238 ± 0·016 (8)	0·043 ± 0·005 (3)	0·045 ± 0·004 (3)	0·031 ± 0·003 (8)	0·031 ± 0·003 (5)
3	3	0·276 ± 0·024 (7)	0·269 ± 0·023 (10)	0·157 ± 0·012 (8)	0·138 ± 0·008 (9)	0·160 ± 0·013 (8)	0·142 ± 0·008 (9)	0·049 ± 0·006 (5)	0·045 ± 0·009 (4)	0·042 ± 0·008 (8)	0·029 ± 0·003 (7)
4	7	0·166 ± 0·011 (8)	0·129 ± 0·015 (8)	0·101 ± 0·013 (9)	0·096 ± 0·010 (10)	0·127 ± 0·027 (8)	0·080 ± 0·008 (9)	0·039 ± 0·005 (6)	0·035 ± 0·003 (6)	0·047 ± 0·016 (8)	0·039 ± 0·014 (7)
5	14	0·105 ± 0·017 (9)	0·069 ± 0·009 (9)	0·058 ± 0·007 (8)	0·054 ± 0·007 (9)	0·059 ± 0·008 (8)	0·044 ± 0·003 (10)	0·028 ± 0·004 (7)	0·028 ± 0·004 (6)	0·017 ± 0·002 (8)	0·015 ± 0·001 (10)

* T and NT denote, respectively, tumour-bearing and non-tumour-bearing mice. For details about the presentation of the results see Table 1.

tumour-bearing or non-tumour-bearing mice. The difference between the ^{65}Zn contents of the tumours and the 'normal' mammary gland tissues removed from the same animal is statistically significant, the value of P being less than 0·01 and very little greater than 0·001. If the results for group 1 (containing the mice killed 3 hr. after the injection of ^{65}Zn) are excluded, the difference is still greater, the value for P then being a little less than 0·001.

With all the groups studied, except group 6, the mean values for the ^{65}Zn contents of the 'normal' mammary gland tissue of the injected tumour mice were less than those for the corresponding non-tumour-bearing mice, but the differences were not statistically significant, the value for P being between 0·1 and 0·2.

For reasons given earlier in this paper there were few tumour mice available for examination 21 days after the injection, and the results for this small group (group 6) are not included in Table 1. It is of interest, however, that they were in general agreement with those recorded for groups 2–5, the mean values, corresponding to those given in columns 3–5 of Table 1, being 0·032, 0·039 and 0·058 for three, two and four mice, respectively.

^{65}Zn *content of a few other tissues of the injected mice*

The results of the determination of the amount of ^{65}Zn in the blood, liver, lung, kidney and skin of the tumour-bearing and the 'normal' mice, at various intervals after the intraperitoneal injection of ^{65}Zn are given in Table 2. Statistical analysis of these results showed that there was no significant difference (the values of P being 0·2 or more in every case), as far as the blood and the few organs examined are concerned, between the values for the tumour mice and those for the normal mice. There was a similar agreement between the corresponding values for the animals in group 6, the mean values for the ^{65}Zn content (as % injected ^{65}Zn/mg. tissue N) ranging from 0·049 and 0·052 for the livers to 0·012 and 0·012 for the bloods of the tumour and non-tumour mice, respectively.

DISCUSSION

Heath (1949) and Heath & Liquier-Milward (1950) observed that transplanted mammary gland carcinomata and transplanted fibrosarcoma contained more ^{65}Zn (on a weight basis) than did normal mammary gland and striped muscle respectively, 18 hr. after the mice had been given a subcutaneous injection of the isotope. In order to try to obtain a stricter comparison between tumour tissue and 'corresponding normal tissue' in the experiments described in this paper, we have studied a spontaneously occurring tumour and a series of two 'control' tissues, and have also calculated the ^{65}Zn contents in relation to the tissue total nitrogen. We believe that in this way it has been possible to obtain a reasonably accurate assessment of the relative zinc requirements, or capacities for utilizing zinc, of tumour and non-tumour tissues.

Thus the observation that mammary gland tumour tissue takes up and retains considerably more of the injected ^{65}Zn than does normal mammary gland tissue is significant and will be investigated further. The difference is too small to be of value for therapeutic radiological purposes, but it is of interest in relation to the metabolic utilization of zinc. Apart from the presence of zinc in carbonic anhydrase (Keilin & Mann, 1939, 1940) little is known about the tissue constituents with which this metal is associated. Lipovitellin contains fairly firmly bound zinc (Tupper *et al.* 1952, 1954) but this lipoprotein is apparently found only in egg-yolk. Heath (1949) and Heath & Liquier-Milward (1950) obtained evidence that at least some of the zinc in tumour tissue is associated with nucleoprotein in the cell nuclei, but Rosenfeld & Tobias (1951) were unable to find appreciable amounts of ^{65}Zn in the nuclear fractions of liver, kidney, lung and transplanted mammary tumour tissues of mice after the intravenous injection of ^{65}Zn as zinc chloride. Hoch & Vallee (1952) and Vallee, Hoch & Hughes (1954) have obtained evidence for the presence of zinc-containing proteins in human leukocytes.

Our limited study of the distribution of ^{65}Zn in some of the organs of the injected mice did not reveal any significant difference between the tumour-bearing and the non-tumour-bearing animals with respect to the uptake of injected zinc by liver, lung, kidney and skin.

SUMMARY

1. The distribution of ^{65}Zn in mice with spontaneously occurring mammary tumours and in non-tumour-bearing mice has been studied at various intervals after a single intraperitoneal injection of a ^{65}Zn-containing zinc–glycine complex.

2. The tumour tissue had a small but statistically significant higher ^{65}Zn content/(mg. tissue nitrogen) than had 'normal' (apparently non-malignant) mammary gland tissue of the same mouse at 1, 3, 7 and 14 days, but not at 3 hr., after the injection of ^{65}Zn. The mean tumour values 1, 3 and 7 days after the injection were about twice those for normal mammary gland tissue.

3. 'Normal' mammary gland tissue from the injected tumour-bearing mice generally contained a little less ^{65}Zn than did the corresponding tissue from the non-tumour mice, but the difference was not statistically significant.

4. The ^{65}Zn contents of the blood, liver, kidney, lung and skin of the tumour-bearing mice were not

statistically different from the corresponding values obtained with normal mice.

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Actinomycete Metabolism

INCORPORATION OF ^{14}C-LABELLED COMPOUNDS INTO STREPTOMYCIN

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Recent work (Numerof, Gordon, Virgona & O'Brien, 1954) has indicated that streptomycin can be elaborated by *Streptomyces griseus* with D-glucose as sole carbon source. The radioactive labels of added [^{14}C]glycine and acetate were significantly incorporated only in the guanidine side chains. As these carbon atoms arise largely, if not entirely, from carbon dioxide (Hunter, Herbert & Hockenhull, 1954), atoms from the substrate are presumably incorporated only after these have been completely metabolized by the organism. Karow, Peck, Rosenblum & Woodbury (1952) have demonstrated the incorporation of the labelled carbon of [^{14}C]-glucose into streptomycin. They did not, however, submit their product to chemical degradation to determine the distribution of the radioactivity within the molecule. Their results have been considerably extended in our work, and we have shown that glucose is incorporated equally into all three monosaccharide residues in streptomycin. In an endeavour to discover some of the intermediate pathways involved in this process, a study has also been made of the incorporation of [^{14}C]streptamine and *N*-methyl-L-glucosamine into the antibiotic by *Strep. griseus*.

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METHODS

The organism, *Streptomyces griseus, albus mutant* (Dulaney Z. 38), was grown on a soybean medium as described previously (Hockenhull, Fantes, Herbert & Whitehead, 1954; Hunter *et al.* 1954). Unless otherwise stated, cultures were harvested 7 days after inoculation. The techniques used in the isolation of crystalline salts of streptomycin from the broth, and in assaying radioactive products, were described in the latter paper.

Chemical degradation of streptomycin. Under acid conditions such as those specified below, streptomycin is decomposed; the glycoside link binding streptidine to the rest of the molecule is broken. The residual streptobiosamine derivative contains streptose joined by a glycosidic linkage to *N*-methyl-L-glucosamine. Vigorous acid hydrolysis of this material leads to extensive decomposition, and although some *N*-methyl-L-glucosamine may be isolated the streptose is completely destroyed.

Streptomycin sulphate was dissolved in the minimum quantity of N-H_2SO_4 and allowed to stand for 2 days at 37°. An equal volume of methanol was added, and the mixture allowed to stand for a further day, when addition of 1 vol. acetone completed the precipitation of streptidine sulphate. After a few hours at 0°, the colourless crystals were filtered off and recrystallized from a small volume of water. The subsequent degradation of the streptidine sulphate was by the method of Hunter *et al.* (1954).

N-Methyl-L-glucosamine hydrochloride was isolated from the mother liquors by removing first the organic solvents *in vacuo* and then the sulphuric acid by passage down a column of weakly basic anion-exchange resin in the OH^- form (Amberlite IR-4B). After evaporating the eluate and aqueous washings from the column almost to dryness *in vacuo* (or freeze-drying), hydrolysis of the methyl streptobiosaminide dimethyl acetal to *N*-methyl-L-glucosamine was effected by refluxing for 2 hr. with conc. HCl. The hydrolysate was filtered, evaporated to dryness *in vacuo* and then converted into the pentaacetyl derivative, as described for the analogous D compound (Kuehl, Flynn, Holly, Mozingo & Folkers, 1947). The crude acetyl derivative was purified by one crystallization from methanol and then by chromatography on acid-washed alumina (Peter Spence and Co. Widnes, Grade H) prepared by washing with dilute H_2SO_4 to pH 4·0–4·5 and then washing free from sulphate and drying overnight at 160° to reactivate. The substance was added in benzene solution and elution carried out with benzene–chloroform (1:1, v/v) after first washing out impurities with benzene. The solvent was removed from the eluate, whereupon the pentaacetyl-*N*-methyl-L-glucosamine formed beautiful colourless needles from chloroform–ether. The yield was 10–20%, based on streptomycin sulphate. The pentaacetyl derivative could be hydrolysed to *N*-methyl-L-glucosamine hydrochloride by refluxing for 1 hr. with 2·5 N-HCl, cautious evaporation *in vacuo* to dryness and crystallization from ethanol.

Materials. Uniformly labelled [^{14}C]glucose was supplied by the Radiochemical Centre, Amersham, Bucks. Subsequently we used material made in our laboratories from [^{14}C]starch which was prepared by the photosynthetic method of Porter & Martin (1952) using excised tobacco leaves (variety White Burley).

[^{14}C]Streptamine sulphate and [^{14}C]*N*-methyl-L-glucosamine hydrochloride were made by degrading [^{14}C]streptomycin obtained biosynthetically from [^{14}C]glucose, as described below.

myo-Inosose was prepared from inositol by oxidation with *Acetobacter suboxydans* American Collection of Type Culture no. 621 (Posternák, 1952).

Scyllitol was made by sodium amalgam reduction of *myo*-inosose (Posternák, 1941).

RESULTS

Incorporation of [^{14}C]*glucose into streptomycin.* Karow *et al.* (1952) obtained a 1% incorporation of [^{14}C]glucose into streptomycin by including the labelled sugar in the initial medium. We have found that the incorporation of isotope may be increased by 5% by delaying the addition of radioactive material until growth had proceeded for 60 hr., by which time the mycelium had almost attained its maximum dry weight. Although these experiments do not prove conclusively that the improved isotope yield is due principally to this delayed addition, the similarity of the cultural conditions is such as to render the assumption reasonable. In our experiment, $8{\cdot}9 \times 10^{-2}$ m-mole streptomycin with a specific activity of 29 μc/m-mole was produced after fermentation in a medium (40 ml.) to which 50 μc of D-[^{14}C]glucose ($5{\cdot}5 \times 10^{-3}$ m-mole) was added after 60 hr. incubation. However, owing to losses during extraction, etc., only two-thirds of the streptomycin was isolated as the crystalline sulphate.

Degradation of the streptomycin revealed the distribution of the ^{14}C in the various parts of the molecule. Detailed results are shown in Table 1, where the radioactivity of the streptomycin not found in either the streptidine or in the *N*-methyl-L-glucosamine is assumed to have been present in the streptose moiety. It can be seen that, within experimental error, the radioactivity is equally distributed in all parts of the molecule with the exception of the carbons of the guanidine side chains.

Incorporation of N-*methyl-*L-[^{14}C]*glucosamine into streptomycin. Strep. griseus* was again allowed to grow for 60 hr. after inoculation on to the soybean medium (40 ml.). *N*-Methyl-L-[^{14}C]glucosamine hydrochloride (0·139 m-mole) with a specific activity of $23{\cdot}8 \times 10^{-2}$ μc/m-mole was now added, dissolved in a little water. Growth was allowed to continue until the normal harvest time, when it was found that $5{\cdot}5 \times 10^{-2}$ m-mole of streptomycin had been produced with a specific activity of $8{\cdot}7 \times 10^{-2}$ μc/m-mole. This represents a 15·5% incorporation of the radioactive label into the antibiotic molecule. Chemical degradation (see Table 2) showed that the labelling was principally located in the *N*-methyl-L-glucosamine part of the molecule, although there was a not inconsiderable amount of radioactivity also present in the streptidine moiety.

Incorporation of [^{14}C]*streptamine into streptomycin.* Streptamine sulphate (0·181 m-mole) with a specific activity of $20{\cdot}9 \times 10^{-2}$ μc/m-mole was added at 60 hr., and at harvest $7{\cdot}1 \times 10^{-2}$ m-mole of streptomycin had been produced with a specific activity of $1{\cdot}64 \times 10^{-2}$ μc/m-mole. Here the incorporation of ^{14}C was only 3·9 %, less than with glucose. Chemical degradation showed that the label was distributed throughout the molecule, although the largest amount was again found in the *N*-methyl-L-glucosamine portion.

Table 1. *Distribution of the radioactivity in* [^{14}C]-*streptomycin obtained after growing* Strep. griseus *in a medium containing* [^{14}C]*glucose*

Organism grown in soybean medium; 60 hr. after inoculation 5·5 m-mole [^{14}C]glucose (sp. activity 9·1 μc/m-mole) added; culture harvested 7 days after inoculation.

Substance	Specific activity (μc/m-mole)	Specific activity (μc/mg. carbon)
Streptomycin	29	$11{\cdot}6 \times 10^{-2}$
Streptidine	9·9	$10{\cdot}4 \times 10^{-2}$
Streptamine	8·8	$12{\cdot}3 \times 10^{-2}$
Barium carbonate (from guanidine carbons)	0·55	$4{\cdot}5 \times 10^{-2}$
N-Methyl-L-glucosamine	10·1	$12{\cdot}2 \times 10^{-2}$
Streptose (by difference)	9·2	$12{\cdot}8 \times 10^{-2}$

Table 2. *Distribution of the radioactivity in* [^{14}C]-*streptomycin obtained after growing* Strep. griseus *in a medium containing* N-*methyl*-L-[^{14}C]*glucosamine*

Organism grown in soybean medium; 60 hr. after inoculation 0·139 m-mole *N*-methyl-L-[^{14}C]glucosamine (specific activity $23{\cdot}8 \times 10^{-2}$ μc/m-mole) added; cultures harvested 7 days after inoculation.

Substance	Specific activity (μc/m-mole)
Streptomycin	$8{\cdot}7 \times 10^{-2}$
Streptidine	$1{\cdot}42 \times 10^{-2}$
N-Methyl-L-glucosamine	$6{\cdot}4 \times 10^{-2}$
Streptose (by difference)	$0{\cdot}92 \times 10^{-2}$

Table 3. *Distribution of the radioactivity in* [^{14}C]-*streptomycin obtained after growing* Strep. griseus *in a medium containing* [^{14}C]*streptamine*

Organism grown on soybean medium; 60 hr. after inoculation 0·181 m-mole [^{14}C]streptamine (specific activity $20{\cdot}9 \times 10^{-2}$ μc/m-mole) added; cultures harvested 7 days after inoculation.

Substance	Specific activity (μc/m-mole)
Streptomycin	$1{\cdot}64 \times 10^{-2}$
Streptidine	$0{\cdot}45 \times 10^{-2}$
N-Methyl-L-glucosamine	$0{\cdot}83 \times 10^{-2}$
Streptose (by difference)	$0{\cdot}36 \times 10^{-2}$

Table 4. *Depression of streptomycin yield by* myo-*inosose and its reversal by scyllitol*

Cultures grown 144 hr. in 250 ml. flasks (40 ml./flask) on rotary shakers (140 rev./min.) at 28·5°. Medium and inoculum according to Dulaney (1949). Streptomycin was determined as maltol (Boxer, Jelinek & Leghorn, 1947).

Expt.	Time of addition to medium (hr.): *myo*-Inosose (1 mg./ml.)	Time of addition to medium (hr.): Scyllitol (1 mg./ml.)	Streptomycin titre (mean of four flasks) (units/ml.)
1	*—	—	1470
1	48	—	1040
1	72	—	970
1	96	—	1290
2	—	—	1530
2	—	0	1670
2	—	24	1500
2	—	48	1420
2	—	72	1640
2	0	—	750
2	0	48	1550

* —; No addition.

Effect of myo-*inosose and scyllitol on streptomycin formation*

Supplementary experiments with the hexitol corresponding to streptamine, scyllitol, and with its parent ketone, *myo*-inosose, were carried out in an attempt to show that by their effect upon yield they might be intermediates in the biosynthesis.

myo-Inosose (1 mg./ml.), while having no apparent effect on the growth of the organism, had a consistent inhibitory effect on the yield of streptomycin. Table 4 shows the results of a typical experiment. It will be noted that *myo*-inosose had little effect when added at a late stage in the fermentation, but had the greatest effect at the time when streptomycin production was beginning. Several other experiments gave closely similar results. Scyllitol (1 mg./ml.), although it did not significantly increase streptomycin yield, appeared to reverse the inhibition caused by *myo*-inosose.

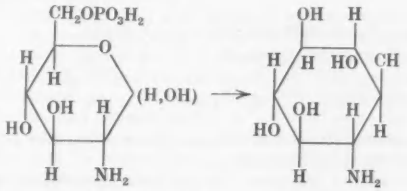
DISCUSSION

From the experiment involving [^{14}C]glucose, combined with the results of Numerof *et al.* (1954), it appears likely that glucose alone can provide *Strep. griseus* with all the carbon atoms it requires for elaborating the streptomycin molecule. The work of Hunter *et al.* (1954) has shown that carbon dioxide for the carbon atoms of the guanidine side chains may be supplied by free carbon dioxide in the gaseous atmosphere and not necessarily by carbon dioxide endogenously produced from glucose or other constituents of the medium. That is to say that free carbon dioxide is, or is in equilibrium with, the form incorporated: in a carbon dioxide-free

atmosphere the guanidine carbon atoms must arise solely from constituents of the medium.

The much greater efficiency of conversion of [^{14}C]glucose into streptomycin obtained by allowing growth to proceed for 60 hr. before adding radioactive material indicates that in the first 2 days of growth under our conditions the glucose is mainly a source of energy. Intermediates are presumably either oxidized completely or used largely for synthesis of mycelium, instead of being accumulated for streptomycin synthesis.

Streptamine does not appear to lie on a direct pathway from glucose to streptomycin, and the incorporation that we did obtain is probably preceded by degradation (possibly deamination to scyllitol or a similar compound. The final proof of the *scyllo* configuration in streptamine is given by Straube-Rieke, Lardy & Anderson, 1953). It is, however, possible that a phosphorylated or other derivative of streptamine occurs as an intermediate in the course of the biosynthesis. Such a compound might arise, for example, by formation of a new C_1–C_6 bond in D-glucosamine 6-phosphate as shown, followed by amination.



Glucosamine 6-phosphate *scyllo*-Inosamine

The strikingly efficient incorporation of *N*-methyl-L-glucosamine into streptomycin makes it likely that this compound, or a near derivative, is normally produced by *Strep. griseus* before the formation of the glycoside link with the rest of the streptomycin molecule.

The degradative evidence also revealed that *N*-methyl-L-glucosamine serves as a more efficient intermediate than glucose for the formation of the streptidine portion of the molecule. This gives rise to conjecture as to the mode of formation of L sugars by the organism. If *N*-methyl-L-glucosamine can undergo ring closure to yield a hexitol derivative, one can also visualize the reverse reaction taking place. Indeed, scyllitol and some of its derivatives are *meso* compounds, and on a purely 'theoretical' basis might be expected to split to both D and L products. Thus, we may postulate the existence of such a biosynthetic chain in *Strep. griseus* as

D-glucose derivative ⇌ scyllitol derivative
⇓
L-glucose derivative
⇓
N-methyl-L-glucosamine.

It is possible that some of the derivatives taking part in such a sequence will be the phosphate esters. It is also possible that the derivatives implicated throughout may be the amines (*scyllo*-inosamine, and L- and D-glucosamines).

The precise nature of the substances involved must await the outcome of further work. We do suggest, however, that the inversion of the D to the L sugar is effected by way of a hexitol intermediate.

An alternative suggestion has been made that the D-glucose → L-glucose transformation may come about by the action of a 'Waldenase' such as converts glucose 1-phosphate into galactose 1-phosphate by inversion at $C_{(4)}$ (Caputto, Leloir, Trucco, Cardini & Paladini, 1949). For this it would be necessary either to start with L sugars or D-idose, or to perform more than one inversion. For reasons of elegance and economy, as it accounts for the formation of both hexitols and L-glucose, we believe that our theory is to be preferred.

The failure of the mould to incorporate streptamine directly into the molecule indicates that it is not on the direct route to streptomycin. Although the experiment to test this has not yet been carried out, it is unlikely that streptidine is formed directly by guanidine transfer without the intermediacy of a diamino moiety. It is more likely that partially synthesized streptamine in the form of a modified scyllitol is first incorporated in the large molecule and that the final stages of synthesis are then carried out.

It is too early to explain the inhibition of streptomycin synthesis by inosose and the reversal of this effect by scyllitol. At least two possibilities do, however, exist: (*a*) interference with ring closure or ring fission in the D-glucose → cyclitol → L-glucose type of synthesis; (*b*) interference with the incorporation of the cyclized moiety into the streptomycin molecule.

SUMMARY

1. A 5% incorporation of D-[^{14}C]glucose into streptomycin by *Streptomyces griseus* has been obtained. This is 5 times the previous maximum (Karow *et al.* 1952) and is thought to be due mainly to delaying the addition of labelled material until the organism had almost attained its maximum bulk (60 hr. after inoculation).

2. Chemical degradation has shown that, within the limits of experimental error, the ^{14}C labels from uniformly labelled [^{14}C]glucose are distributed equally between the streptamine, streptose and *N*-methyl-L-glucosamine parts of the molecule. The carbons of the guanidine side chains contain much less radioactivity than the average of the other carbon atoms, which is consistent with the hypothesis that these carbons are incorporated only secondarily by way of carbon dioxide.

3. Streptamine is not converted into streptomycin as efficiently as is glucose.

4. *N*-Methyl-L-[^{14}C]glucosamine, added after the organism had grown for 60 hr., was incorporated into streptomycin in over 15% yield. The radioactivity was located largely in the corresponding part of the antibiotic molecule, but the L sugar was also converted into the streptidine moiety more efficiently than D-glucose.

5. It is suggested that the inversion of D-glucose to give L sugars proceeds by way of a hexitol-type intermediate.

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Creatine and Carbohydrate Metabolism

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In a study of the distribution of high energy phosphates in X-ray irradiated guinea pigs it was observed that a 24 hr. fast produced a marked rise in the liver creatine of control and irradiated animals. Although a considerable literature exists on the consequences of starvation on creatine distribution (Benedict, 1907; Hunter, 1928; Beard, 1943), so far as we are aware creatinuria is reported to occur in prolonged rather than short-term deprivation. It is generally accepted that the creatine arises as muscle protein is broken down (Mendel & Rose, 1911; Myers & Fine, 1913), but since in our experiments a significant elevation was obtained at a time when peripheral tissue breakdown is unlikely (cf. Addis, Poo & Lew, 1936*a*, *b*) we thought it worth while to investigate the mechanism of the effect.

METHODS

Animals. Guinea pigs were fed a liberal diet of cabbage and bran. Rats from the department strain of Wistar rats were fed a stock diet of rat cubes (Messrs Heygate and Son, Northampton). During starvation water was always available *ad lib.* Adrenalectomized rats were maintained on 1% saline. The completeness of removal of adrenals was checked at death. Thyroid deficiency was produced by giving 0·1% thiouracil in the drinking water for 6 weeks before testing. The glands were sectioned and examined *post mortem* by Dr R. V. Coxon and histological changes associated with chronic thiouracil-induced hypothyroidism were found to be present.

Hind-limb perfusion. The rats were anaesthetized with ether. The viscera and testes were removed and the descending aorta was cannulated above the bifurcation. The animal was transected and the hind limbs were connected to the perfusion apparatus. The carcass was supported on a muslin ring in a glass funnel; this served to filter and collect the venous return. The funnel was covered with a Perspex lid. The perfusion fluid was warmed to 38° and the limbs were kept at about 38° by an infrared lamp. Perfusion fluid (60–100 ml.) was oxygenated with a Hooker oxygenator and recirculated by means of a pump designed by Dr E. H. Schuster for use in the Department of Pharmacology, Oxford. The perfusion fluid was blood diluted with an equal volume of Ringer–phosphate and contained 10 mg. heparin/100 ml. The blood was obtained from donor rats by cardiac puncture under ether. The perfusion fluid could be sampled and the pressure and flow rate measured as required.

Liver extract. The animals were killed by cervical fracture, the abdomen was opened and the liver dropped immediately into ice-cold 10% (w/v) trichloroacetic acid (TCA) and dispersed in a high-speed homogenizer. The residue after centrifuging was extracted twice with 6% (w/v) TCA and the combined extracts were made up to volume.

Blood extract. For creatine estimations the animals were decapitated, the blood was collected in 10% TCA and the precipitated protein re-extracted with TCA as above. For assay of glucose, blood was deproteinized in the cadmium sulphate reagent of Fugita & Iwataka (1931).

Muscle extract. The animals were anaesthetized with sodium pentobarbitone and the quadriceps femoris of both legs displayed. Samples (200–500 mg.) were quickly dropped into liquid air, then dispersed in 10% TCA and re-extracted as usual. Some specimens were powdered in a steel mortar before homogenization.

Urine samples. Pairs of rats were placed in metabolism cages and the urine was collected. Because of the formation of creatine from creatinine at room and body temperatures, experimental collections were always compared with those from the same animals under similar control conditions.

Creatine. This was estimated by the method of Ennor & Stocken (1953). Bound creatine (phosphocreatine) was given by the difference between free creatine and that obtained after hydrolysis of the neutralized TCA extract by heating for 10 min. at 65° with 0·1 N-H_2SO_4. Recoveries of creatine added to the samples were checked both before and after hydrolysis (cf. Barker, Ennor & Harcourt, 1950).

Liver glycogen. This was estimated by precipitation from the ice-cold TCA extracts by an equal volume of ice-cold ethanol. After coagulation, the precipitate was centrifuged, dissolved in N-H_2SO_4, hydrolysed for 2–2·5 hr. at 100° and the sugar estimated on a neutralized sample by the method of Hagedorn & Jensen (1923). The factor 0·927 was used to convert glucose into anhydrous glycogen.

Blood glucose. This was estimated by the method of Hagedorn & Jensen (1923).

Liver arginase. This was estimated by the method of Folley & Greenbaum (1946). No activators were used. The urea was converted into NH_3 by urease–Dunning tablets and the NH_3 estimated in the Markham (1942) apparatus.

Chromatography. Ascending chromatograms of the ether-extracted and concentrated liver extracts were run as described for amino acids by Consden, Gordon & Martin (1944) and for creatine and glycocyamine by Ames & Risley (1948).

RESULTS

The effect of starvation on the creatine and phosphocreatine content of guinea pig livers is shown in Table 1. The total creatine level in fed guinea pigs appeared to be less than 12 mg./100 g. liver (wet wt.) in both male and female animals. A 12 hr. fast produced a rise in total creatine content which was maintained after 24 and 48 hr. starvation. The increase was also observed in X-ray-irradiated animals, and in all the groups there was a proportionate increase in bound creatine so that the percentage phosphorylated remained fairly constant. This constancy in the percentage phosphorylated, and the development of maximal colour within 20 min. in the Barritt reaction suggested that the increase in creatine-reacting substance was creatine itself, and this was confirmed using the specific creatine micro-organism (Ennor & Stocken, 1953), when 96–97% of the total creatine-reacting substance was destroyed.

The proportionate increase in liver phosphocreatine when the total creatine content increased, and the stability of the adenosine triphosphate (ATP) level at this time (Rowles, 1951), suggested that the creatine–phosphocreatine system responded directly to the creatine concentration and was not limited by ATP.

The nitrogen content of guinea pig livers increased from 3 to 3·5% (wet wt.) as a result of a 24 hr. fast, but the rise in creatine cannot be accounted for solely as a concentration effect. It has been shown that of many mammalian livers the guinea pig has the greatest ability for synthesis of creatine (Cohen, 1951), and one possible explanation for the rise could be an increased synthesis of creatine in the liver from nitrogen precursors made available in the course of gluconeogenesis. Chromatographic examination of the liver extracts, however, showed no qualitative difference in amino acid contents of the livers from fed and starved guinea pigs, and no obvious increase in possible amino acid precursors of creatine (glycine, arginine, glutamic acid). Development of the papers by the Jaffé reaction to demonstrate creatine-like compounds (Ames & Risley, 1948) confirmed the increase of creatine in starvation but failed to show any increase in glycocyamine.

Since protein deprivation is known to affect liver enzymes (Miller, 1948, 1950), the concentration of liver arginase was compared in fed and starved guinea pigs (Table 2). The enzyme activity expressed on a nitrogen basis (236 and 244 units/g. N) was unaltered so that diversion of arginine metabolism towards creatine synthesis appeared improbable.

Table 1. *Mean creatine levels in the livers of fed and starved adult guinea pigs*

No. of animals and sex	Period of starvation (hr.)	Creatine (mg./100 g. wet wt. of liver)			Bound creatine as % of total
		Free	Total	Bound	
2 M	0	8·1	11	2·8	25
2 F	0	7·4	9·5	2·1	22
1 M	12	28	33	5	15
5 M	24	32	44	12	28
2 F	24	—	26	—	—
2 M	48	20	32	12	38
4 M*	24	34	48	14	29
4 F*	24	54	71	17	24
					Mean 26

* Starvation begun 6 days after X-ray irradiation with 1000 r.

Because these experiments provided no conclusive evidence regarding liver creatine synthesis in starvation, it was decided to investigate more closely the conditions under which creatine appeared in the liver. The work of Cathcart (1909) and many others had established that the creatinuria of fasting was prevented specifically by carbohydrate feeding. It was therefore decided to investigate simultaneously the creatine and glucose or glycogen levels in blood and liver and the urinary creatine excretion in animals in acute starvation in an attempt to discover the cause of the altered creatine distribution. For this purpose rats were used and it will be seen (Table 3) that the liver creatine level in rats is lower than that in guinea pigs, but a 24 hr. fast still produced an increase which was in fact detectable at 12 hr. and remained steadily elevated over the period of starvation studied. Simultaneous determination of blood creatine showed that this also increased sharply as a result of 24 hr. starvation and then fell to a steady level about 30% above that in fed animals. The excretion of creatine appeared to reflect the blood creatine level, rising very steeply to a peak after about 30 hr. fasting and then falling somewhat. The liver glycogen levels were similar to those reported previously for male rats in starvation (Harrison, 1953) and the absence of TCA-extractable glycogen as 12–24 hr. (Bloom, Lewis, Schumpert & Shen, 1951) coincided with the rise of creatine in blood and liver. The re-appearance of glycogen, after 2–3 days starvation, did not reduce the liver creatine level as might have been expected if the glycogen level was the determining factor.

In a further series of experiments blood glucose and creatine levels in four female rats were simultaneously determined over a 30 hr. period of starvation (Table 4). Blood samples were obtained by cardiac puncture under ether. The blood glucose fell markedly between 6 and 24 hr. starvation, rising again by 30 hr. To avoid the complication due to the effect of ether on the blood glucose level the experiment was also carried out with a group of male rats, half the animals being killed to give the fed glucose and creatine levels and the remainder being deprived of food for 24 hr. before death. The creatine levels were elevated after 24 hr., and again there was a pronounced drop in blood glucose concentration. The results of these experiments and of those shown in Table 3 suggested that the fall in blood glucose level apparent after 24 hr. starvation might be the initiating factor in the appearance of increased liver creatine and creatinuria.

Table 2. *Arginase levels in the livers of fed and starved adult male guinea pigs*

Amounts recorded are for 100 g. wet wt. of liver. An arginase unit = the amount of enzyme liberating 1 mg. urea in 30 min. under the conditions described by Folley & Greenbaum (1946).

	Total creatine (mg.)	Arginase (units)
Fed	15·4	778
	10·0	660
	12·8	683
Starved	33·3	953
	43·1	830
	41·4	783

Table 3. *Tissue creatine levels in starved rats*

Three groups, *A*, *B* and *C*, of rats were used. The animals in group *A* had been adrenalectomized; those in groups *B* and *C* were controls. In each group urine was collected from a pair of rats from the same litter as those used for tissue analyses.

Period of starvation (hr.)	Weight loss (%)	Liver glycogen (mg./100 g. wet wt.)		Liver creatine (mg./100 g. wet wt.)			Blood creatine (mg./100 ml.)	Period of collection of urine	Urinary excretion of creatine (μg./rat/hr.)		
	C	*B*	*C*	*B*	*C*	*A*	*C*	(hr.)	*B*	*C*	*A*
0	0	5250	5025	3·4	3·0	4·1	2·5	0–6	9	13	30
		5580	3855	4·2	3·5	4·8	3·4				
6	—	1720	—	3·6	—	5·6	—	6–12	14	—	35
		2380	—	2·8	—	4·0					
12	—	3	—	5·0	—	5·8	—	12–24	22	—	132
		4	—	5·3	—	5·7					
24	7	0	47	7·2	5·4	8·0	6·4	24–30	153	361	445
	6	0	10	7·4	5·2	6·7	6·3	30–36	193	—	505
								36–48	158	—	268
48	15	456	53	5·2	6·3	8·2	6·3	48–54	—	233	191
	11	890	26	6·1	4·7	8·3	5·5	—	—	193	—
72	17	—	332	—	5·2	8·6	3·4	72–78	—	147	—
	17	—	161	—	5·3	—	4·2	—	—	—	—
96	24	—	492	—	6·8	—	4·2	—	—	—	—
120	26	—	252	—	5·9	—	4·0	—	—	—	—
	27	—	640	—	5·9	—	4·6	—	—	—	—

Table 4. *Effect of starvation on blood glucose and creatine levels in rats*

Blood samples (0·5 ml.) were obtained from the female rats by cardiac puncture under ether. The male rats were killed to provide blood samples.

	Period of starvation (hr.)				Period of starvation (hr.)			
	0	6	24	30	0	6	24	30
Sex	Creatine (mg./100 ml. blood)				Glucose (mg./100 ml. blood)			
F	3·5	3·7	4·2	3·5	143	110	72	127
F	2·6	3·9	5·4	7·2	107	124	65	97
F	2·9	3·6	3·0	3·8	130	124	70	127
F	2·8	3·3	*	—	125	124	—	—
M	3·9	—	5·8	—	108	—	77	—
M	4·1	—	7·0	—	94	—	65	—
M	3·7	—	5·6	—	88	—	61	—
M	3·8	—	6·1	—	98	—	64	—

* Died from haemorrhage following cardiac puncture.

Table 5. *Effect of 24 hr. starvation on the creatine content of the quadriceps femoris of the male rat*

	Creatine (mg./100 g. wet wt.)			
	Free	Total	Bound	Percentage phosphorylated
	Fed rats			
	270	522	252	48
	226	431	205	48
	240	541	301	56
	217	422	205	49
	315	489	174	36
	289	463	174	38
Mean	260±38	478±48	218	46
	Starved rats			
	320	563	243	43
	296	480	184	38
	210	391	181	46
	277	564	287	51
	276	495	219	44
	303	464	161	35
Mean	280±38	493±65	213	43

As no evidence had been found for enhanced creatine synthesis in the liver after 24 hr. starvation, mobilization of creatine from peripheral sites was an obvious alternative explanation. Skeletal muscle creatine and phosphocreatine were therefore measured after 24 hr. starvation when the rise in blood and liver creatines was well established. After 24 hr. there appeared to be a decrease in the percentage creatine phosphorylated (Table 5) but the levels of free and total creatine were not significantly different ($P = 0·5$). It is clear, however, that since the concentration of total creatine in muscle is a hundred times that in blood, a trivial change in the proportion of free creatine could, if released into the circulation, bring about a very marked increase in the blood concentration.

Because direct analytical techniques were inadequate to demonstrate either the origin of the labile creatine or the controlling factors in its mobilization, it was decided to turn to perfusion experiments. With the co-operation of Dr R. B. Fisher and Mr D. Lindsay the possibility of an increased creatine leak from heart muscle in starvation was investigated by means of their perfused rat heart preparation (Bleehen & Fisher, 1954). With hearts from normal well-fed rats there was no difference in total creatine liberated into the perfusion fluid after 30 min. when galactose replaced glucose in the medium. Hearts from 24 hr. starved rats gave the same loss of creatine when perfused either with or without glucose in the medium as had been found for the normal hearts.

Since no demonstrable difference was obtained with the heart muscle, hind limb perfusions were undertaken. We are indebted to Dr E. Bulbring for teaching us the operative technique. In these experiments the limbs were perfused with recirculating fluid and the venous outflow was sampled for glucose and creatine content. The hind limbs were weighed before and after the experiments (initial weights 60–100 g.) and haematocrits were measured on samples of the perfusion fluid. The initial and final volumes of blood, the volume removed in sampling and the rate of loss of water by evaporation from the animal and supporting muslin were measured so that it was possible to establish complete balance sheets for the water and creatine changes in the experiments.

The first runs demonstrated that glucose was removed from the circulating fluid by the preparation and that as the glucose concentration fell creatine appeared in the perfusion fluid (Fig. 1). In a typical experiment 58 mg. glucose disappeared and 2·5 mg. creatine appeared in the circulating fluid after 2 hr. Of the glucose disappearing, a control experiment showed that approximately 12 mg. were taken up by the red cells in 2 hr. Since the leakage of creatine under these conditions might be due to the progressively deteriorating preparation, reversal of the phenomenon was attempted by

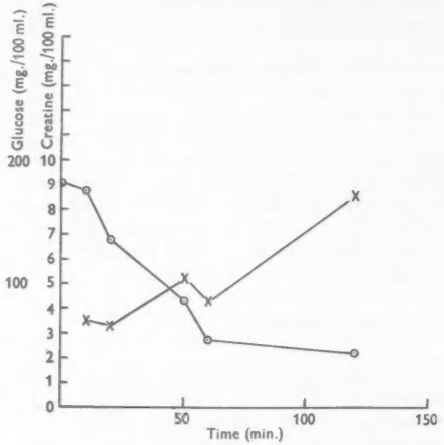

Fig. 1. The effect of falling glucose concentration on the creatine level in rat hind limb perfusate. Initial volume of perfusion fluid 94 ml. Initial weight of hind quarters 100 g., final weight 113 g. Constant haematocrit 23%. ⊙, Glucose; ×, creatine.

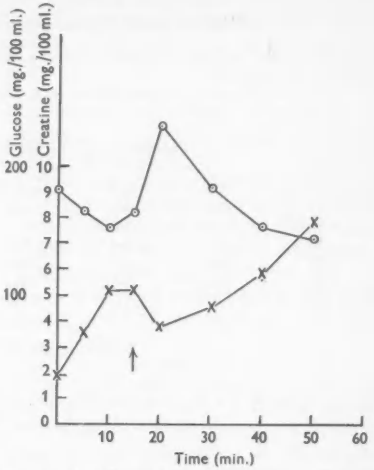

Fig. 3. The effect of sodium iodoacetate on the creatine level in rat hind limb perfusate. Initial volume of perfusion fluid 100 ml. Initial weight of hind quarters 103 g., final weight 116 g. Initial haematocrit 23%, final 25%. ⊙, glucose; ×, creatine. At 15 min. 18·6 mg. sodium iodoacetate and 50 mg. glucose added in 10 ml. perfusion fluid.

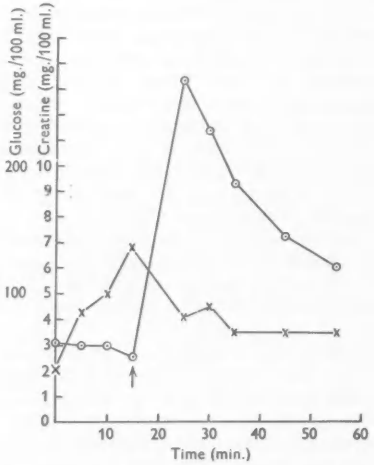

Fig. 2. The effect of introducing glucose on the creatine level in rat hind limb perfusate. Initial volume of perfusion fluid 100 ml. Initial weight of hind quarters 90 g., final weight 101 g. Initial haematocrit 21·5%, final 24%. ⊙, glucose; ×, creatine. At 15 min. 150 mg. glucose added to perfusion fluid reservoir.

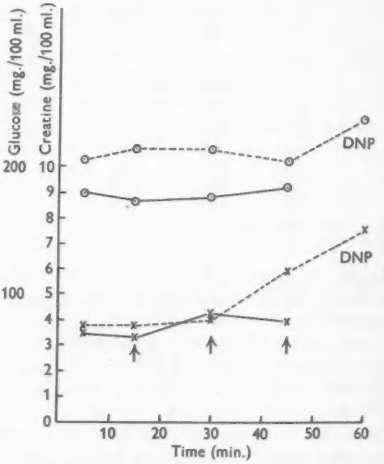

Fig. 4. The effect of dinitrophenol (DNP) on the creatine level in rat hind limb perfusate.

⊙—⊙, glucose; ×—×, creatine. Control. 90 ml. perfusion fluid. Initial weight of hind quarters 69 g., final weight 98 g. Initial haematocrit 30%, final 39%. At 15 and 30 min. 50 mg. glucose added in 1 ml. perfusion fluid.

⊙- - -⊙, glucose; ×- - - -×, creatine. Initial weight of hind quarters 60 g., final weight 76 g. Initial haematocrit 25%, final 32%. At 15 min. 18·4 mg. DNP and 50 mg. glucose added in 1 ml. fluid; at 30 and 45 min. 50 mg. glucose added.

increasing the glucose concentration in the circulating fluid after an initial period at a low level (Fig. 2). The limbs were perfused for 15 min. with 100 ml. 'blood' unsupplemented by glucose; as much as possible of the fluid was then collected and 150 mg. glucose dissolved in it before reintroducing the 'blood' into the circulation. There was an immediate drop in creatine concentration in the perfusion fluid which was maintained as glucose utilization continued. This reversal of the creatine leak from the preparation was almost invariably seen when glucose was added to the perfusion fluid, but was most marked when both the blood and the hind limbs were from fed animals.

To demonstrate the importance of glucose utilization in the maintenance of muscle creatine the effects of inhibition of glucose oxidation were investigated. The introduction of sodium iodoacetate did not prevent glucose disappearance but produced a striking increase in creatine level (Fig. 3, cf. Fig. 2). At the completion of the experiment the limbs were completely rigid.

In an experiment with dinitrophenol (DNP) in which the circulating glucose level was kept constant by the introduction of 50 mg. of glucose in 1 ml. 'blood' at 15 min. intervals, creatine once more escaped into the circulation (Fig. 4). This result is in contrast to a similar control experiment without DNP, in which there was a constant creatine concentration, and calculation showed that 0·6 mg. creatine disappeared from the perfusion fluid. With DNP the limbs were slightly rigid at the end of the experiment.

As a result of the perfusion experiments it seemed that reversible loss of creatine from skeletal muscle could be produced by a fall in blood sugar from 200 mg./100 ml. to 50 mg./100 ml. In view of the influence of endocrines on carbohydrate metabolism, a few experiments have been carried out to investigate possible hormonal effects on creatine distribution. In these experiments creatine mobilization in starvation was followed by determining urinary creatine excretions in fed and fasted control and experimental animals.

Adrenalectomized rats were examined in some detail (Tables 3, 6). The creatine excretion from fed adrenalectomized rats tended to exceed that in the controls, as had been reported earlier by Bodansky & Duff (1936). After 24 hr. fasting the creatinuria was extremely high and this was paralleled by the rapidly and greatly elevated liver creatine level. In the one animal examined after 3 days' starvation the liver creatine level was still very high, although its creatine excretion had dropped sharply. Renal failure was tentatively assumed. These results with adrenalectomized rats were not surprising in view of the known disturbance in glucose metabolism.

Thyroprivic and hypophysectomized rats also exhibited the creatinuria of starvation, the hypothyroid animals being rather more sensitive than the controls. The sensitivity of the hypothyroid rats was interesting, as Bodansky & Duff (1936) reported a creatinuria and decreased myocardial and skeletal muscle creatine levels in fed hyperthyroid rats.

Some experiments on both rats and guinea pigs were also carried out to investigate the effect of cold stress. Short exposure to the cold had no effect on either fed or starved animals (Table 7), whereas Browne, Karady & Selye (1939) found that 24 hr. exposures produced a creatinuria.

Table 6. *Effect of hormonal withdrawal on urinary creatine levels of rats*

Urine was collected for 6 hr. Results are expressed as μg. creatine/rat/hr. The period of starvation was 24 hr.

Controls		Adrenalectomized	
Fed	Starved	Fed	Starved
4	179	20	469
3	234	20	518
8	265	12	597
2	202		
2	253		

Hypophysectomized		Hypothyroid	
Fed	Starved	Fed	Starved
5	162	53	499
4	296	8	360
36	180		
26	83		

Table 7. *Effect of cold stress on liver creatine*

Stress was applied by keeping rats for 1 hr. and guinea pigs for 1·5 hr. in the cold room at 2°, the light being kept on. Animals were killed directly after stress. Control animals were kept in another room. Food was removed from both groups for the stress period.

Condition	Wt. of animal (g.)	Liver creatine (mg./100 g. wet wt.)	Liver glycogen (g./100 g. wet wt.)
	Fed rats		
Control	160	3·4	4·0
	138	4·3	4·0
	119	5·2	2·6
Stressed	200	4·4	3·4
	128	5·3	2·6
	122	5·5	3·3
	120		2·9
	Rats starved for 24 hr.		
Control	200	5·8	—
	200	8·0	—
Stressed	200	6·2	—
	200	8·4	
	Fed guinea pigs		
Control	530	7·2	1·1
Stressed	510	7·1	2·6

DISCUSSION

The experiments reported here followed the finding of a markedly increased liver creatine content in starved guinea pigs, an observation which has also been made by Ennor (personal communication). The creatine content of the livers of fed guinea pigs corresponded to that reported by Ennor & Rosenberg (1952) and is very low; 24 hr. fasting produced a threefold increase in total creatine content and a proportional increase in phosphocreatine, so that the amount of this form of high-energy phosphate showed an absolute increase. Only two simultaneous determinations of blood and liver creatines were performed with guinea pigs (the values in mg./100 g. or ml. were—fed, liver creatine 3 and 8; blood 2 and 2: starved, liver creatine 27 and 44; blood 2 and 5), but it is interesting that in this species there was a concentration of creatine in the liver in starvation, whereas in rats the liver creatine was never significantly different from the blood level and the increase on a nitrogen basis, after a 24 hr. fast, was less marked. In rats, therefore, the creatinuria which was established after about 24 hr. starvation was the most convenient index of creatine mobilization.

It is known (Pitts, 1934) that in dogs a small rise in plasma creatine produces a creatinuria and if the same consideration holds for rats this is adequate to explain the observed increased creatine excretion. Nevertheless, some derangement of kidney function as a result of starvation cannot be excluded as a contributary factor.

Although the increase in guinea pig liver creatine and the creatinuria in rats was striking, nevertheless the absolute quantities involved are very small. In a 200 g. rat the creatine excreted in the 30 hr. following withdrawal of food is not more than 0·4% of the total muscle creatine (muscle provides 45% of the body weight) and neither in rats nor in guinea pigs was any change in muscle creatine detected between fed and 24 hr. starved animals.

In explaining the cause of creatine mobilization many workers have concluded that carbohydrate metabolism is an important factor, but some of the experimental results are conflicting. Thus, Hobson (1939) found that a diet rich in carbohydrate was associated with a creatinuria, whereas Mendel & Rose (1911) prevented the creatinuria of starvation by feeding a carbohydrate diet. Factors contributing to the divergence of results could be the lack of sensitivity in the method employed for assay and failure to minimize the conversion of creatinine into creatine during the time between voiding and analysis of the urine.

The intimate relation between glucose and creatine is directly supported by the coincidence of creatinuria with a fall in blood sugar below 100 mg./100 ml. and would explain the greater creatine excretion in starved adrenalectomized rats. Tissue analyses did not reveal the origin of the creatine, but while perfused heart failed to show any response to differences in availability of glucose, the hind limb preparation was quite sensitive. Rat skeletal muscle lost creatine reversibly with a fall in circulating glucose from 150 to 50 mg./100 ml., and factors known to interfere with glucose utilization, although possibly affecting creatine phosphokinase itself (Ennor & Rosenberg, 1954), produced a loss of creatine from muscle. With the perfusion fluid used in these experiments it was not possible to follow changes in blood potassium and phosphate levels, although these might be expected to parallel the creatine transport.

The reversible dependence of muscle creatine on the blood glucose level indicates that creatine can be released from this tissue in conditions of sudden carbohydrate shortage. It is suggested that the reduction in availability of oxidizable substrate will reduce the amount of creatine phosphorylated and the resultant increase in cellular creatine concentration will allow the liberation of small amounts of creatine into the circulation. In view of the reversible loss of creatine in hind limb perfusion experiments it is likely that liberation of creatine precedes that classically associated with muscle protein breakdown.

Disturbances in carbohydrate metabolism may also explain some of the effects on creatine distribution which have been reported in adrenalectomized animals. Our fed adrenalectomized rats, which were not used until at least 1 week after operation and which were maintained on saline, ordinarily had a slightly elevated liver creatine and creatinuria. No muscle creatine estimations were performed, but Conway & Hingerty (1953) have reported an increase in muscle phosphocreatine 5 days after adrenalectomy accompanied by a reduction in glucose 6-phosphate and an increase in ATP.

The influence of the parathyroids was not investigated, but Imrie & Jenkinson (1932) and Brown & Imrie (1932) have shown in cats that the phosphate retention following creatine administration is increased by parathormone administration, and that in thyroparathyroidectomized animals phosphocreatine resynthesis in muscle after stimulation is much reduced.

An additional factor influencing the release of creatine might be an alteration in electrolyte balance. Our experiments with hypertonic perfusion fluids were inconclusive since glucose utilization was impaired, but it is possible that some of the results reported by Beard (1943) can be explained on the basis of electrolyte imbalance and/or tissue dehydration.

SUMMARY

1. A two- to threefold increase in guinea pig liver creatine occurs within 24 hr. after the withdrawal of food.

2. In rats the increase in liver creatine is less marked, but a pronounced creatinuria occurs coincident with a fall in blood sugar to 60–70 mg./100 ml.

3. Perfusion experiments have demonstrated that rat skeletal muscle creatine is reversibly sensitive to blood sugar levels.

4. The relation between creatine distribution, carbohydrate metabolism and electrolyte balance is discussed.

We are indebted to Professor J. H. Burn for advice and for extending to us the facilities of his Department, to Drs Coxon, Fisher and Parsons for much helpful discussion and to Mrs Lawrence for providing us with hypophysectomized rats. We should also like to thank Miss M. Cole and Mrs J. Kingerlee for skilled technical assistance.

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Uridine Compounds in Glucuronic Acid Metabolism

2. THE ISOLATION AND STRUCTURE OF 'URIDINE-DIPHOSPHATE-GLUCURONIC ACID'

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We have previously reported that liver contains a nucleotide composed of uridylic acid, labile phosphate and glucuronic acid in equimolecular proportions, and that the incubation of this substance with *o*-aminophenol or menthol in the presence of broken-cell liver suspensions results in the formation of the corresponding glucuronides (glucosiduronic acids) (Dutton & Storey, 1951, 1953). The enzyme system in the liver suspensions and some properties of the nucleotide have been described (Dutton & Storey, 1954).

The present paper deals with the isolation of the pure nucleotide and a study of its degradation products under various conditions. The results confirm an earlier suggestion (Dutton & Storey, 1953) that its structure resembles the uridine diphosphate compounds isolated by Caputto, Leloir, Cardini & Paladini (1950), Cabib, Leloir & Cardini (1953) and Park (1952*a*, *b*), and that it may be regarded as 'uridine-diphosphate-glucuronic acid'.

Since the new nucleotide appears to be a substrate in the enzymic process, throughout both this and the preceding paper (Dutton & Storey, 1954) we have used the term 'factor' rather than 'cofactor' or 'coenzyme', which imply a function which is, as yet, unproved.

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EXPERIMENTAL

Methods

Measurement of activity of factor and of glucuronide synthesis. The course of the purification of the factor was followed by measuring its activity in the liver-suspension system in presence of *o*-aminophenol or (−)-menthol as previously described (Dutton & Storey, 1954). Recently, it was found advantageous to reduce all volumes to approx. one-fifth, to permit the use of smaller amounts of the factor. Aminotrishydroxymethylmethane (THAM) buffer was used in preference to phosphate, since it does not form a precipitate with Mg^{2+}, and glucuronide synthesis appeared to be equally good in both media. Each reaction vessel received 0·04 ml. 0·5M THAM buffer (pH 7·4) containing 0·15M-$MgCl_2$, the final incubation volume being 0·6 ml. The 1 cm. microcells of the Spekker absorptiometer were used in reading the extinctions.

Phosphorus determinations. Inorganic P was determined by the method of Fiske & Subbarow (1925). For labile P, the sample was made N with HCl and heated at 100° for the required period; inorganic P was then determined. Total P was estimated by the method of King (1946). These methods could be used satisfactorily for measuring 0·1–2·0 μg. P by suitably reducing the volumes of all solutions.

Uridylic acid. This was measured from its extinction at 260 mμ. in 1 cm. cells with a Unicam SP. 500 spectrophotometer, using the data of Smith & Markham (1950).

Glucuronic acid. Glucuronic acid in the pure factor was determined by the naphthoresorcinol reaction as modified by Paul (1951; see also Dutton & Storey, 1954), but the ethyl acetate extraction was omitted. D-Glucurone (Roche Products) was used as the standard.

Reducing power. This was measured by the method of Folin & Malmros (1929).

Paper chromatography. This was carried out in all-glass tanks by the ascending method whenever possible, but if greater resolution was required the descending method was used. Except where specifically stated, chromatograms were run at room temperature. Whatman no. 541 paper was chosen for its low ash content, and it was satisfactory with all solvent systems without any preliminary washing. Ultraviolet photography of developed chromatograms was carried out as described by Markham & Smith (1949, 1951). Phosphate compounds were detected with the molybdate reagent of Hanes & Isherwood (1949). Identifications of spots were made by reference to standard compounds rather than by measurement of R_F values.

Materials

Uridine 5′-phosphate and uridine 5′-pyrophosphate were synthetic products kindly given by Professor Sir Alexander Todd, F.R.S., and Dr G. W. Kenner. Yeast uridylic acid (L. Light and Co.) and cytidylic acid (a gift from Dr C. M. Mauritzen) were presumably mixtures of the 3′ and 2′ isomers, but for convenience they will be referred to as the 3′-phosphates. Adenosine 5′-phosphate (L. Light and Co.) was pure as judged by paper chromatography and determinations of total P and absorption at 260 mμ.

α- and β-D-glucuronic acid 1-phosphates, as previously described (Dutton & Storey, 1954), were gifts from Dr C. A. Marsh and Dr O. Touster, respectively. α-D-Glucose 1-phosphate was presented by Dr A. B. Roy.

Dried cobra (*Naia naia*) venom and antiserum were the materials described by Johnson, Kaye, Hems & Krebs (1953), and were generously presented by Dr M. A. G. Kaye.

Russell's viper venom. 'Stypven' (Burroughs Wellcome and Co.) was used.

RESULTS

Isolation of the pure factor

Extraction from liver. The procedure already described (Dutton & Storey, 1954) for obtaining a crude preparation of the factor was modified by extracting the disrupted rabbit liver with boiling water (50–80 ml./100 g. liver) rather than with a solution of trichloroacetic acid, to avoid any possible destruction of the activity by prolonged contact with strong acid. The ice-cold, strained extract was brought to pH 2 with 20% (w/v) trichloroacetic acid. An equal volume of ethanol was then added and the precipitate of glycogen and protein was centrifuged down. This and subsequent operations were carried out with all solutions chilled to 0° whenever possible.

Fractionation of barium salts with ethanol. The solution was immediately brought to pH 8·2–8·5 (glass electrode) with NaOH and fractionated according to procedure *B* of LePage & Umbreit (1945). The barium salts were precipitated by adding 3 ml. M barium acetate/100 g. wet weight of liver, and ethanol to a total concentration of 80% (v/v). After chilling for 30 min. the mixture was centrifuged and the supernatant discarded. The precipitate was dissolved in dilute HCl and the solution readjusted to pH 8·2–8·5. The precipitate of water-insoluble barium salts thus obtained was reprecipitated by dissolving in HCl and readjusting the pH, and 4 vol. of ethanol was added to the combined supernatants from these two precipitations. The active factor was present in the resulting precipitate of water-soluble ethanol-insoluble barium salts. The other barium-salt fractions did not show any activity, either singly or in combination. Barium was subsequently removed as $BaSO_4$, the solution was adjusted to pH 7·4 with NaOH and then concentrated *in vacuo* at 50° to a convenient volume (about 150 ml. for a batch of 7–15 rabbits).

Phenol extraction. The solution had a bright greenish-yellow tint at this point, and unless most of this colouring matter was removed traces were carried through the subsequent purification and were present in the final product. The solution was therefore saturated with phenol and extracted thrice with one-sixth vol. phenol saturated with water, followed by four washings with 0·25 vol. peroxide-free diethyl ether. The ether was then removed *in vacuo* at 50°.

Chromatography on Dowex-1. This step was based on the method of Cohn & Carter (1950) for adenosine phosphates, but to obtain the maximum separation of the factor from ultraviolet-absorbing impurities a different range of eluting solutions were used.

A column (1 cm. × 1 cm.²) of Dowex-1 anion-exchange resin was washed with 2N-HCl until all ultraviolet-absorbing impurities had been removed and then with water until the effluent was neutral. The solution of the factor (150 ml.) was first brought to pH 8·5 with concentrated NH_3 solution (sp.gr. 0·88) and any precipitate removed before it was added to the column at room temperature. The flow rate was kept at 2 ml./min. by pressure from a mercury manometer. Distilled water (40 ml.) was passed through the column, followed by 0·02M-NaCl in 0·01N-HCl until the issuing solution was acid to litmus. No activity was eluted

under these conditions. The following fractions were then collected manually (flow rate 3 ml./min.): 4 × 25 ml. with 0·02 M-NaCl in 0·01 N-HCl as eluent; 8 × 10 ml. with 0·05 M-NaCl in 0·01 N-HCl; 6 × 10 ml. with 0·1 M-NaCl in 0·01 N-HCl and 4 × 10 ml. with 0·2 M-NaCl in 0·01 N-HCl. All these fractions were cooled in ice-water, neutralized immediately, and 0·4 ml. samples tested in the liver-suspension system for the presence of the factor. Since paper chromatography of solutions from the previous stage had suggested a relationship between activity and absorption at 260 mμ., the fractions were also examined for absorption at that wavelength. The relationship between activity and absorption is shown in Fig. 1. The activity in zone *A* suggests the constant elution of a small amount of the factor during the rapid washing out of inactive material. In zone *B*, during the rapid rise to the pronounced peak and the subsequent fall, the activity and absorption maintain a fairly constant ratio.

According to Smith & Markham (1950), purines are liberated from their ribonucleotides by hydrolysis with N-HCl for 1 hr. at 100°, whereas pyrimidine ribonucleotides are virtually unaffected. Hydrolysis of samples of the various pooled fractions under these conditions and subsequent chromatography in *iso*propanol—HCl (Wyatt, 1951), adjusted to give maximum separation of purines and pyrimidines, revealed that absorption in zone *P* was due to adenine compounds (deduced from the identical R_F value of the released purine with authentic adenine, and from its absorption curve after elution (Ploeser & Loring, 1949)), that in zone *A* to a mixture of adenine and pyrimidine nucleotides and in zone *B* almost entirely to pyrimidine nucleotides.

The combined *B* fraction (60 ml.) was concentrated *in vacuo* at 50° to 0·33 vol. and fractionated through the barium salts as described previously to avoid salt effects in the subsequent paper chromatography. The precipitate of water-soluble ethanol-insoluble barium salts was then freed of Ba^{2+} and ethanol and concentrated *in vacuo* at 30° to 2–3 ml. of colourless solution.

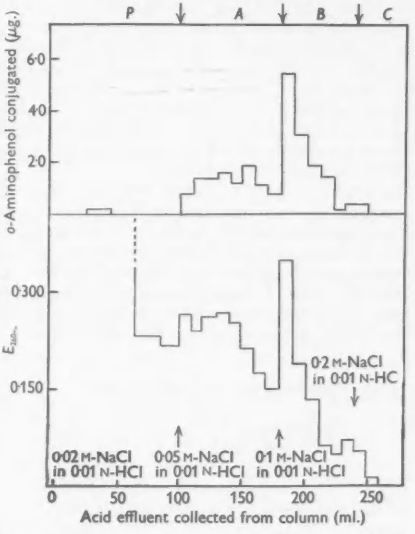

Fig. 1. Ion-exchange chromatography of water-soluble ethanol-insoluble barium salts from rabbit liver, showing the relationship between ultraviolet absorption (lower diagram) and glucuronide formation (upper diagram) in acid effluent from Dowex-1 column.

Paper chromatography. When a band of this solution was chromatographed by the ascending method in methanol–water–conc. (0·88) NH_3 (60:30:10, by vol.) (Bandurski & Axelrod, 1951) for 18 hr. at 2°, only one ultraviolet-absorbing zone was apparent, and this coincided with the activity. Chromatography in the acid solvent of the same authors, methanol–formic acid (88%, w/v)–water (80:15:5, by vol.), gave rise to two bands with strong ultraviolet absorption, and only the more slowly moving one contained the factor. (A third, very weak band of absorption was sometimes detectable between the two other bands.) However, the use of a strongly acid solvent resulted in a considerable loss of activity and poorly defined bands, presumably owing to decomposition of the factor during chromatography. These difficulties were avoided with a weakly acidic solvent system. After development for 18 hr. at 2° in ethanol–M ammonium acetate, pH 5·5 (60:40, by vol.), the slower-moving band was cut out and eluted with water by capillarity. When all the material had been put through this process the factor was freed of ammonium acetate by precipitation as the Ba salt. It was then converted into the potassium salt and stored at −20°, at which temperature it was stable for long periods.

Various preparations of the slower-moving band behaved as a single substance in the following solvents at 2° (all proportions are by vol.): *n*-propanol–concentrated NH_3–water (60:10:30) (Hanes & Isherwood, 1949); methanol–conc. NH_3–water (60:10:30); *iso*propanol–$(NH_4)_2SO_4$ (1% w/v) (60:40) (Anand, Clark, Hall & Todd, 1952); ethanol–M ammonium acetate, pH 4·0 (60:40); methanol–formic acid (88%, w/v)–water (80:5:15). Consistent analytical results (see later) were obtained with several different preparations of this band, and there does not appear to be any evidence for the presence of appreciable amounts of contaminating materials.

Comments on the method. Since it was essential to remove the livers immediately after the death of the rabbits (Dutton & Storey, 1954), it was usual to prepare boiled extracts from about six livers at a time, whereas the remainder of the purification was more conveniently carried through with several such batches. Later, the ion-exchange procedure was modified for handling increased amounts of material by the use of a larger column (3 cm. × 1·8 cm.²) and different volumes of eluting solutions. For the material from fifteen rabbits (150 ml.), once the effluent was acid 150 ml. of 0·02 M-NaCl in 0·01 N-HCl and 150 ml. of 0·05 M-NaCl in 0·01 N-HCl preceded the 3 × 20 ml. of 0·1 M-NaCl in 0·01 N-HCl required to elute the factor, the flow rates remaining as before. To minimize losses on the column the process was carried out as rapidly as possible, and the factor was in contact with the resin for only 3 or 4 hr. It seems probable that failure to observe this condition was the cause of the difficulties recently reported with this procedure (Smith & Mills, 1954). After the column of resin had been regenerated with 2 N-HCl, it could be used again.

Yield. In a preparation from thirty rabbits (approx. 2·5 kg. liver), 65% of the activity of the boiled aqueous extract was recovered after the barium–ethanol fractionation and 20% after the resin treatment. The final yield was 15% and amounted to about 8 mg. of the pure factor.

Whereas the weight of the pure factor was determined by measurement of the absorption at 260 mμ., the percentages are only approximate, since they were derived by enzymic assay. In general, the weight of the factor isolated represented 10–20% of the boiled-extract activity. The extraction of the finely minced tissue with boiling water was itself very inefficient, but the volume of water had to be severely restricted to keep operations within the limits of the facilities available. Indeed, much larger amounts of the factor are present in liver than the above figures suggest. Direct assays of boiled extracts of rabbit and mouse liver, using a high water–tissue ratio of 10:1, gave values of the order of 20 mg. (34 μmoles) factor/100 g. liver, when a correction is made for the transfer of the glucuronic acid from the factor to the *o*-aminophenol being only 50% complete (see later). This figure is in good agreement with that quoted by Smith & Mills (1954) for guinea pig liver, if a similar correction were applicable to their conditions of assay.

Where the pure factor was not required, the acetone-precipitated 'crude factor' (Dutton & Storey, 1954), or a similar preparation made from a boiled extract, has been found useful for many purposes. A subsequent barium–ethanol treatment gave further purification without great loss of active material.

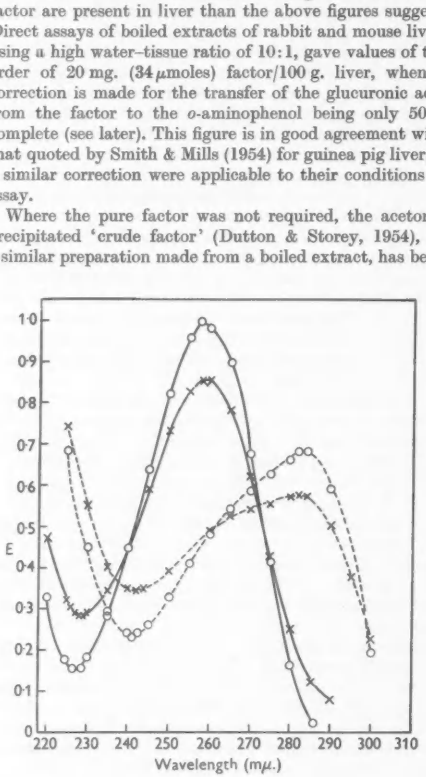

Fig. 2. Absorption spectrum of uracil (circles) and of pyrimidine from factor (crosses) in 0·1 N-HCl (full lines) and in 0·1 N-KOH (broken lines).

Investigations on the structure of the factor

Presence of uracil. As has already been indicated, on paper chromatograms the region which showed activity always coincided with that having ultraviolet absorption. The material eluted from this area exhibited an absorption spectrum resembling that of a uridine-containing nucleotide (Ploeser & Loring, 1949; Caputto *et al.* 1950) with a maximum at 261 mμ. and minimum at 229–230 mμ. in 0·1 N-HCl.

To prepare the free pyrimidine, the solution was transferred to a heavy-walled glass tube and blown to dryness at 70°. Formic acid (0·35 ml. of 98%, w/v) was added and the tube was sealed. After heating at 175° (5 hr. were found necessary to free the base completely, cf. Chargaff *et al.* 1950), the solution was chromatographed by the method of Wyatt (1951) with adenine, guanine, cytosine and uracil as markers. The only ultraviolet-absorbing area was at the uracil level, and after elution it gave the characteristic uracil absorption curves (Hotchkiss, 1948; Ploeser & Loring, 1949) with maxima and minima in acid and alkaline solutions virtually identical with those of authentic uracil (Fig. 2). Since uracil was the only ultraviolet-absorbing component in the factor, and since it was present as uridylic acid (see later), the measurement of the absorption at 260 mμ. was used in calculating the concentration of solutions of the factor, using the value $\epsilon_{260\ m\mu.} = 9890$ for uridylic acid (Smith & Markham, 1950); all molar ratios for the other constituents (Table 1) were based on the assumption that the factor contained one molecule of uridylic acid.

Phosphate. After the pure factor had been run as a band for 18 hr. at 2° in methanol–conc. NH_3–water (60:10:30, by vol.), longitudinal strips were sprayed for phosphate and the remainder of the chromatogram was cut latitudinally into narrow equal strips, which were then added to the liver-suspension system in a complementary series of flasks. The position of the strips giving rise to glucuronide formation corresponded exactly with the phosphate-containing zone and with the ultraviolet absorption.

The factor was found to contain two molecules of phosphorus per molecule of uridylic acid (Table 1), and when it was heated in N-HCl at 100° half the phosphorus was liberated in the inorganic form ('labile P') in 10 min., whilst the remainder was released much more slowly (Fig. 3).

Glucuronic acid. A definite, positive naphthoresorcinol reaction was not obtained with crude preparations before ion-exchange chromatography, but afterwards it was

Table 1. *Molar ratios of various components of the factor referred to uridylic acid*

	Molar ratio (uridylic acid = 1·00)				
Preparation no.	1	2	3	4	5
Total P	2·12	2·00	—	—	2·15
Labile P	1·10	0·90	—	—	1·07
Glucuronic acid (naphthoresorcinol reaction)	0·88	0·91	—	—	—
Reducing substance (calc. as glucuronic acid)	—	—	0·92	0·95	—

readily evident. With paper chromatography, the area giving the reaction always coincided with the zone of ultraviolet absorption, phosphate and activity. The naphthoresorcinol-positive substance behaved identically with authentic glucuronic acid in paper chromatography and ionophoresis experiments. Different preparations showed a constant proportion of approximately one molecule of glucuronic acid to one of uridylic acid (Table 1). An identical ratio was found with the orcinol method of Meijbaum (LePage & Umbreit, 1945), using D-glucurone as the standard. Uridine 5′-pyrophosphate did not give any reaction for pentose under these conditions.

Reducing substance. It has been previously shown (Dutton & Storey, 1954) that the activity of the factor is completely lost after heating at 100° for 8 min. in 0·1 N-HCl. Now Caputto *et al.* (1950) found that treatment of uridine-diphosphate-glucose with 0·01 N acid at 100° for 5 min. split off the glucose quantitatively. When the pure factor was heated for 10 min. in 0·1 N-HCl at 100°, a reducing substance was likewise liberated in an amount equivalent to one molecular proportion, when calculated as glucuronic acid (Table 1). The reducing value was not increased by further heating.

Products of hydrolysis by N *acid*

As has been shown above, when the factor was heated with N-HCl at 100° for 15 min. one molecule of inorganic phosphate was liberated. The other breakdown products were examined by paper chromatography.

After hydrolysis of the factor in N-H_2SO_4 at 100° for 15 min., the solution was cooled and brought to pH 3–4 with $Ba(OH)_2$. The $BaSO_4$ was centrifuged down and washed, and the combined supernatants were neutralized with 0·1 N-KOH before concentrating to a small volume *in vacuo* at 25–30°. The solution was then chromatographed with untreated factor, uridine 5′-pyrophosphate, uridine 5′-phosphate and uridine 3′-phosphate for comparison (Fig. 4*a*, *b*). The ethanol–boric acid system slowed down the uridine 5′-phosphate very markedly compared with the 3′ isomer, presumably because of complex formation (cf. Cohen & Scott, 1950). In the two solvent systems shown, and also in *iso*propanol–$(NH_4)_2SO_4$ (1%, w/v) (60:40, by vol.), the only ultraviolet-absorbing substance present in the hydrolysate travelled exactly as did uridine 5′-phosphate.

When chromatograms of the hydrolysate in ethanol–M ammonium acetate, pH 5·5 (60:40, by vol.) were treated with the aniline oxalate reagent (Partridge, 1949), an orange spot was found at the level of glucurone and a faint smear at that of glucuronic acid. Formation of the lactone would be expected during the N acid hydrolysis if glucuronic acid were present. Galacturonic acid did not form a lactone under such conditions (cf. Partridge, 1948), and gave only a brown spot at the glucuronic acid level.

It thus appeared that treatment of the factor with N-H_2SO_4 for 15 min. at 100° gave rise to uridine 5′-phosphate, inorganic phosphate and glucuronic acid only. No other compounds containing phosphorus, showing ultraviolet absorption or giving a colour with aniline oxalate were detected.

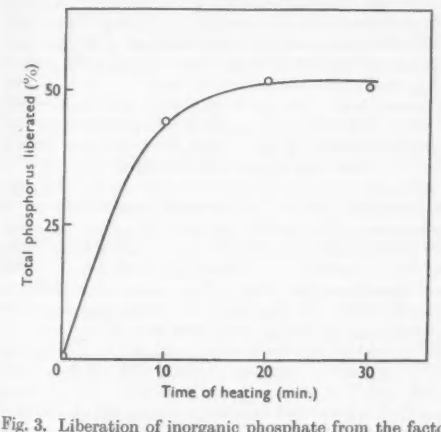

Fig. 3. Liberation of inorganic phosphate from the factor on heating in N-HCl at 100° for varying periods of time.

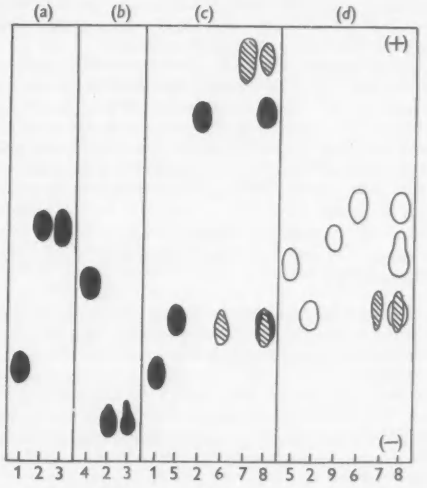

Fig. 4. Paper chromatography and ionophoresis of hydrolysis products of the factor. All origins at base line. Chromatogram solvents allowed to drip off paper. Ultraviolet-absorbing areas are black, areas giving colour with aniline oxalate are cross-hatched and those showing phosphate are unshaded. (*a*) Chromatography in ethanol–M ammonium acetate, pH 4·0 (60:40, by vol.) for 17 hr. (*b*) Chromatography in ethanol–boric acid (0·6%, w/v) (80:20, by vol.) for 17 hr. (*c*) Chromatography in ethanol–M ammonium acetate, pH 4·5 (65:35, by vol.) for 20 hr. (*d*) Ionophoresis in 0·05 M sodium acetate, pH 5·9, at potential of 3·5 v/cm. for 18 hr. 1, pure factor; 2, uridine 5′-phosphate; 3, product of hydrolysis of factor with N-H_2SO_4 at 100° for 15 min.; 4, uridine 3′-phosphate; 5, uridine 5′-pyrophosphate; 6, β-D-glucuronic acid 1-phosphate; 7, glucuronic acid (potassium salt, Corn Products Ltd.); 8, products of hydrolysis of factor with 0·1 N-H_2SO_4 at 100° for 7 min.; 9, orthophosphate.

Further identification of uridine 5′-phosphate

The uridylic acid was prepared by hydrolysing the factor in N-HCl for 15 min. at 100° and treating with barium acetate at pH 8·2. After removal of the inorganic phosphate, the nucleotide was precipitated with ethanol and converted into the K salt by treatment with K_2SO_4. The solution was then concentrated *in vacuo*.

Hydrolysis by Russell's viper venom. Gulland & Jackson (1938) first showed that the venom of Russell's viper and certain other snakes was a potent source of an enzyme that hydrolysed 5′-nucleotides, but that it was without effect on 3′-nucleotides. Such venoms have been subsequently used (Park, 1952*b*; Cabib *et al.* 1953; Cabib & Leloir, 1954) to prove that the uridylic and guanylic acids isolated from the corresponding diphospho-glycosyl compounds are 5′-phosphates. In our experiments adenosine 5′-phosphate was completely hydrolysed by Russell's viper venom, whilst uridine 5′-phosphate and the uridylic acid from the factor were hydrolysed to the extent of approximately 60–95%. Cytidine 3′-phosphate and uridine 3′-phosphate were not hydrolysed even after 2 hr. incubation. Two results are shown in Table 2. They show clearly that the uridylic acid from the factor resembles the nucleotides with the phosphate esterified at the 5′ position.

Copper-complex formation. The micromethod described by Caputto *et al.* (1950) and by Park (1952*b*) was adapted for amounts of nucleotide of the order of 2% of that used by these authors. The sample (0·5 ml. containing 45–80 μm-moles nucleotide) was allowed to react, with occasional stirring, for 2 hr. in a 3 ml. microcentrifuge tube with 0·1 ml. 10% (w/v) $Na_2PO_4, 12H_2O$ and 0·3 mg. copper phosphate. After centrifuging, 0·3 ml. of the supernatant was mixed with 0·1 ml. 0·1% (w/v) diethyl dithiocarbamate and the colour extracted by shaking with 0·7 ml. amyl alcohol. Any turbidity in the upper layer was removed by adding 0·05 ml. ethanol and mixing carefully with a capillary pipette. The colours were read in the Spekker absorptiometer using Ilford filter no. 601 (transmitting 385–470 mμ.).

The results showed some variation probably owing to the small scale of working, but nevertheless the uridylic acid from the factor closely resembled adenosine and uridine 5′-phosphates in its copper-binding capacity (Table 3). The 3′-phosphates were unable to solubilize appreciable amounts of copper.

Table 2. *Action of Russell's viper venom upon uridylic acid from the factor and upon other nucleotides*

Each tube contained 12 μg. venom, 0·05 ml. 0·5 M THAM buffer, pH 7·40 and substrate (final concentration in each case about 0·1 mM) in a total volume of 0·35 ml. Incubation was for 1 hr. at 37°. The reaction was stopped with 0·1 ml. trichloroacetic acid (20%, w/v) and 0·05 ml. of each of the Fiske & Subbarow (1925) reagents were then added. In the controls venom was added after incubation. Phosphate standards were carried through the complete procedure.

Substrate	P liberated (%) Expt. 1	Expt. 2
Adenosine 5′-phosphate	100	—
Uridine 5′-phosphate	73	71
Uridylic acid (from factor)	82	58
Uridine 3′-phosphate	0	—
Cytidine 3′-phosphate	0	0

Table 3. *Copper-binding capacity of various nucleotides*

Nucleotide	μm-moles Cu bound/μm-mole nucleotide
Adenosine 5′-phosphate	0·59
Uridine 5′-phosphate	0·35
Uridylic acid (from factor)	0·38
Uridine 3′-phosphate	0·05
Cytidine 3′-phosphate	0·00

Products of hydrolysis by 0·1 N acid

A solution of the factor was heated at 100° in 0·1 N-H_2SO_4 for the 7 min. necessary for complete inactivation (cf. Dutton & Storey, 1951, 1954), and it was then neutralized and concentrated as previously. Chromatography in ethanol–M ammonium acetate, pH 4·5 (65:35, by vol.) showed two ultraviolet-absorbing areas corresponding with uridine 5′-phosphate and uridine 5′-pyrophosphate (Fig. 4*c*) in the ratio of approximately 1:0·8, and a trace of inorganic phosphate. In addition, the aniline oxalate reagent revealed two areas corresponding with glucuronic acid 1-phosphate and glucuronic acid in rate of travel and shade of colour (pink and brown, respectively). Both areas gave a positive naphthoresorcinol reaction for glucuronic acid, with a ratio of approximately 1:0·8. Unfortunately, the slower glucuronic acid 1-phosphate spot coincided exactly with uridine 5′-pyrophosphate in this and several other solvents, so that the area contained more phosphorus than corresponded with the ultraviolet absorption.

A more satisfactory separation of the hydrolysis products was obtained by paper ionophoresis in an apparatus of the Durrum (1950) type using Whatman no. 541 paper. After the paper had been sprayed with 0·05 M sodium acetate buffer, pH 5·9, a potential of approximately 3·5 v/cm. was applied for 18 hr. Examination of the strip for phosphate showed the following compounds, in order of increasing rate of travel: uridine 5′-phosphate, uridine 5′-pyrophosphate, inorganic phosphate (a trace) and glucuronic acid 1-phosphate (Fig. 4*d*). With aniline oxalate, the orange-brown spot of glucuronic acid was noted at the uridine 5′-phosphate level, and the characteristic rosy-pink of glucuronic acid 1-phosphate coincided with the fastest-moving phosphate-containing area. Both these regions gave a positive Tollens naphthoresorcinol reaction.

Although it was not feasible to characterize the glucuronic acid phosphate further, other considerations confirmed its formulation as the 1-ester. An acyl phosphate linkage at C-6 would also have been easily hydrolysable, but it would not have explained the increase in reducing power after acid hydrolysis (see later). The recent observation of Schmitz, Potter, Hurlbert & White (1954), that during the ion-exchange chromatography of the acid-soluble fraction of rat liver on Dowex-1 formate resin uridine 5′-pyrophosphate and a substance apparently identical with the factor were eluted together, also indicated that the carboxyl group must be free since otherwise they would not have had similar net charges. Positions 2–4 seem highly improbable, as such phosphate esters would presumably be relatively stable to hydrolysis in N acid. It has not yet been possible to show

whether the glucuronic acid 1-phosphate is the α or the β isomer, since they travelled at identical rates in ionophoresis experiments over a considerable pH range, and with paper chromatography any difference was too small to be demonstrated with certainty.

Since uridine 5′-pyrophosphate, uridine 5′-monophosphate and glucuronic acid 1-phosphate were identified as products of mild acid hydrolysis of the factor, then it follows that breakdown must have taken place at two different bonds, since there are only two molecules of P/molecule of uridylic acid. The only structure which will satisfy all the experimental findings is one in which uridine 5′-phosphate is joined to the glucuronic acid 1-phosphate through a pyrophosphate linkage. This type of structure could account satisfactorily for the slow destruction of the activity of the factor even with large amounts of a purified alkaline phosphatase preparation, whereas it was destroyed rapidly by crude tissue suspensions (Dutton & Storey, 1954).

Inactivation by snake venoms

Confirmatory evidence for the proposed structure was sought in a study of the action of certain snake venoms on the factor. Kornberg & Pricer (1950) have described a nucleotide pyrophosphatase in potatoes, but the preparation of the purified enzyme appeared difficult. A similar enzyme which split diphosphopyridine nucleotide (Chain, 1939; Zeller, 1951) and certain dinucleotide pyrophosphates (Christie, Elmore, Kenner, Todd & Weymouth, 1953) was demonstrated in various snake venoms. Cobra (*Naia naia*) venom contains a nucleotide pyrophosphatase splitting both diphosphopyridine nucleotide and coenzyme A (personal communication from Dr M. A. G. Kaye). We have therefore studied the action on the factor of Russell's viper and cobra venoms, neither of which liberated inorganic phosphate from α-glucose 1-phosphate or the α- and β-glucuronic acid 1-phosphates. The results of these experiments confirmed the structure proposed for the factor. Both Russell's viper and cobra venoms destroyed the activity of the factor on incubation at pH 7·4 in THAM buffer containing Mg^{2+} for 30 min. at 37°. Liberation of inorganic phosphate was much slower (Table 4), and was always less than 50% of the total phosphate, strongly suggesting that cleavage first took place at a pyrophosphate bond and that the liberated uridine 5′-phosphate was acted upon by the 5′-nucleotidase present. A similar, incomplete liberation of phosphate was observed when uridine 5′-phosphate itself was the substrate. The release of phosphate from both the factor and uridine 5′-phosphate was inhibited by antiserum, which was found by Johnson *et al.* (1953) to affect the 5′-nucleotidase more strongly than the pyrophosphatase. Support for the idea that a glucuronic acid 1-phosphate was liberated initially was provided in Expt. 3 of Table 4. After the 150 min. hydrolysis period 'labile P' was determined on the digest. The 'labile P' liberated (25·2 μm-moles) was in good agreement with the theoretical (27·6 μm-moles), showing that the P liberated by the enzymic hydrolysis (24·2 μm-moles) must have corresponded with the acid-stable P.

Inactivation by alkali

We have previously found (Dutton & Storey, 1954) that the factor is inactivated when heated with 0·1 N acid or alkali for 8 min. at 100°. In the present paper we have described the quantitative liberation of a reducing substance from the factor on heating at 100° with 0·1 N-HCl for 10 min. However, it was found that even without a preliminary acid hydrolysis the factor still showed 75% of the total reducing value; but since an approximately equal loss of activity was brought about merely by heating under the alkaline conditions (0·022 M-Na_2CO_3) used in the Folin & Malmros (1929) determination of reducing power, this result was not necessarily at variance with the proposed structure of the factor, where the glucuronic acid is linked in the 1-position. If the reducing value was determined after first heating with 0·1 N-KOH for 10 min. at 100° it was less than that of the untreated material and even subsequent acid hydrolysis (0·1 N-HCl for 8 min. at 100°) did not raise it to the maximum attainable value. This diminution in the rate of release of reducing substance by acid after alkaline treatment was also observed by Caputto *et al.* (1950) and Paladini & Leloir (1952) with uridine-diphosphate-glucose (UDPG). These authors presented evidence for the formation of uridine 5′-phosphate and a cyclic glucose monophosphate, probably esterified at positions 1 and 2, as the primary decomposition products. With the factor it has not yet been possible to show that a glucuronic acid phosphate ester is split off. After 4 hr. at 15° or 30 sec. at 100° in 9 N-NH_3 there was no chromatographic alteration detectable in the factor when run in the ethanol–M ammonium acetate, pH 7·5 (75:30, by vol.) solvent of Paladini & Leloir (1952) and subsequently examined for phosphate. In the ethanol–concentrated NH_3 (75:30, by vol.) solvent of the same authors the factor also gave only a single spot. Nevertheless, the factor and UDPG are both inactivated when chromatographed at room temperature in ammoniacal solvents. Further investigations of the action of alkali were postponed until larger amounts of the factor were available.

Table 4. *Liberation of inorganic phosphate from the factor by the action of snake venoms*

Each tube received 0·5 M THAM buffer, pH 7·4 (0·04 ml.) containing 0·015 M-$MgCl_2$, with other additions as shown, the total volume being 0·46 ml. Incubation was at 37°. The remainder of the procedure was as in Table 2.

	Russell's viper venom		Cobra venom					
	Expt. 1	Expt. 2	Expt. 3			Expt. 4		
Factor (μm-moles)	55·2	31·1	27·6			27·6		
Venom (μg.)	13	25	5			5		
Time of incubation (min.)	40	100	60	150	150	90	210	210
Antiserum (μg.)	—	—	—	—	20	—	—	20
P released (μm-moles)	23·0	20·4	21·6	24·2	15·5	11·6	16·5	0
Uridylic acid hydrolysed (%)	42	66	78	88	56	41	60	0

Nature of the reaction

In a previous paper (Dutton & Storey, 1954) we described some features of the reaction which took place in presence of the factor, an acceptor (*o*-aminophenol or (−)-menthol) and a liver suspension. In contrast with glucuronide formation in liver slices (Storey, 1950), the reaction was anaerobic and was unaffected by cyanide. It was also very rapid, being about 75% complete within 15 min. We have furthermore found that, provided the *o*-aminophenol is in large (approximately fivefold) excess, the amount of glucuronide formed is proportional to the concentration of the factor, indicating that the factor is a substrate rather than a cofactor. Since the factor contains glucuronic acid, it seems likely that the reaction involves merely the transfer of glucuronic acid from the factor to the acceptor (ROH) according to the equation:

$$\text{UDPglucuronic acid} + \text{ROH} \rightarrow \text{UDP} + \text{R–O—glucuronic acid},$$

where UDPglucuronic acid, UDP and R–O—glucuronic acid represent the factor, uridine 5′-pyrophosphate and the glucuronide, respectively.

Unfortunately, further investigation of the nature of the reaction was greatly hindered by the labile nature of the enzyme system and its association with insoluble cytoplasmic particles (Dutton & Storey, 1954). Only crude tissue preparations could therefore be used, either a whole-liver suspension or the supernatant prepared from this by centrifuging for 5 min. at 700 *g* ('centrifuged suspension'). The amount of glucuronide formed was only 40–60% (average 50%) of that required by the above equation, but as it could not be increased appreciably by varying the reaction conditions, it is unlikely that an equilibrium was being reached. This was further investigated by incubating UDP (0·05 mM) and *o*-aminophenylglucuronide (0·07 mM) with the centrifuged suspension in presence of saccharate (0·1 mM) as a β-glucuronidase inhibitor (cf. Dutton & Storey, 1954), but it was not possible to detect any disappearance of the glucuronide. The low yield of glucuronide might at least partially be explained by destruction of the factor by the liver suspension, since it lost 30% of its activity in 8 min. and 60% in 20 min. when incubated with the centrifuged suspension in the absence of *o*-aminophenol. The only degradation product which showed ultraviolet absorption and was detectable on paper chromatograms was uridine. UDP itself was apparently completely degraded to uridine with great rapidity even by preparations of the insoluble particles alone.

DISCUSSION

Although a full chemical characterization of the factor was impracticable with the amounts of material available, the present results appear to show without much doubt that it is a nucleotide composed of uridine 5′-phosphate and glucuronic acid 1-phosphate linked by a pyrophosphate bond. Our earlier proposal (Dutton & Storey, 1953) that it was structurally analogous to UDPG and uridine-diphosphate-*N*-acetylglucosamine (UDPAG) is thus confirmed, and it may therefore be regarded as 'uridine-diphosphate-glucuronic acid' (UDPglucuronic acid). Its presence in liver has been recently confirmed by Smith & Mills (1954) and by Hurlbert & Potter (1954).

Smith & Mills (1954) found that UDPglucuronic acid did not undergo the pyrophosphate fission that was observed with UDPG and UDPAG (cf. Kalckar, 1953), neither did any synthesis take place from uridine triphosphate and glucuronic acid 1-phosphate. They therefore considered that the structure of UDPglucuronic acid might not be strictly analogous to UDPG and UDPAG. Our results make this reservation unnecessary and the differences in enzymic behaviour are more probably explicable in terms of substrate specificity. We ourselves also obtained negative results in attempting to demonstrate synthesis of the factor by incubating α- or β-glucuronic acid 1-phosphate, UDP and adenosine triphosphate with liver suspensions (cf. Trucco, 1951). The possibility exists that the configuration of the glucuronic acid–phosphate bond in UDPglucuronic acid might differ from that in UDPG, which most probably is α (Paladini & Leloir, 1952). In the recently described syntheses of trehalose (Leloir & Cabib, 1953) and of sucrose (Leloir & Cardini, 1953) the UDPG gives rise to an α-glucoside. All the naturally occurring glucuronides have the β configuration, but in our present state of knowledge it is not possible to conclude that the configuration of the UDPglucuronic acid is also β, since inversion might occur during the formation of the glycosidic bond. This has been shown to take place during the phosphorolysis of maltose by certain bacterial extracts (Fitting & Doudoroff, 1952).

Although it is not certain that the glucuronyl transfer from the UDP to the acceptor represents the final stage of glucuronide synthesis *in vivo* or in liver slices, the available evidence suggests this to be true. Both UDPglucuronic acid and the enzyme system have so far been detected only in the liver (Dutton & Storey, 1954), whilst with tissue slices the liver, and to a very minor extent the kidney, were the only organs tested which showed activity (Storey, 1950). In liver slices and in suspensions the formation of glucuronides was inhibited by 'glucuronate' prepared from glucurone by treating it with a solution of sodium bicarbonate, but not when sodium hydroxide was used instead (Storey, 1950; Dutton & Storey, 1954; cf. Sie & Fishman, 1954). Previous work has also shown that β-glucuronidase is not concerned in glucuronide formation, either in the intact animal and whole-cell preparations (Levvy, 1952, 1953), or in the present system with broken-cell suspensions (Dutton & Storey, 1954).

Storey (1950) suggested that the final step of glucuronide synthesis in liver slices might be the reaction of glucuronic acid 1-phosphate with the acceptor under the influence of a phosphorylase.

However, all experiments in which the α- and β-glucuronic acid 1-phosphates were added to liver suspensions in presence of *o*-aminophenol were negative (Dutton & Storey, 1954). It is now fairly clear that the glucuronyl donor is not a simple phosphate ester but one which can be regarded as a uridyl-substituted glucuronic acid 1-phosphate. The reaction proposed represents a new type for the formation of glycosidic bonds, since unlike the well-known phosphorolytic reactions inorganic phosphate does not take part (cf. Kalckar, 1953). The syntheses of trehalose (Leloir & Cabib, 1953) and of sucrose (Leloir & Cardini, 1953) are also examples of UDP acting as a glycosyl donor. The sucrose reaction was reversible, but we have not succeeded in showing this for glucuronyl transfer. These reactions are all to be distinguished from the pyrophosphate cleavages in which uridyl transfer takes place (Kalckar, 1953, 1954).

Although Leloir and his co-workers showed originally that UDPG was the coenzyme of galactowaldenase (Caputto *et al.* 1950), the presence of the nucleotide in yeast unadapted to galactose and in animal tissues led them to conclude that this was not its only function. The isolation of an acetylaminouronic acid derivative of UDP (Park, 1952*b*), of UDPAG (Cabib *et al.* 1953), of UDPglucuronic acid (Dutton & Storey, 1953; and present paper), of guanosine-diphosphate-mannose (Cabib & Leloir, 1954) and of UDP-acetylgalactosamine (Videla, 1954), and the apparently wide distribution of at least certain of these in animal tissues (Caputto *et al.* 1950; Rutter & Hansen, 1953; Schmitz *et al.* 1954; Smith & Mills, 1954) and in plants (Buchanan *et al.* 1952) has led to speculation as to their possible role in polysaccharide synthesis. The results of Leloir & Cabib (1953), Leloir & Cardini (1953) and probably those of the present authors show that certain UDP compounds can donate glycosyl groups with the formation of a glycosidic linkage; but whether this process is extended to the synthesis of such mucopolysaccharides as hyaluronic acid, chondroitin sulphuric acid and heparin, which are built up mainly from acetylglucosamine, acetylgalactosamine and glucuronic acid units, is as yet unknown. An obvious difficulty from this view point is that UDPglucuronic acid and the conjugating enzyme system have so far not been demonstrated with certainty in any tissue other than the liver, but one possibility might be that this organ has a specific function in forming a disaccharide unit, *N*-acetylhexosamine glucuronide, as suggested by Smith & Mills (1954).

If UDPglucuronic acid is indeed concerned in the synthesis of mucopolysaccharides, then we may well inquire whether glucuronide formation does not represent a diversion from the normal course of glucuronic acid metabolism, and whether glucuronide-forming compounds (for which virtually the sole structural requirement appears to be an hydroxyl group) should not be regarded merely as trapping agents for active glucuronic acid, just as aromatic amines trap active acetyl groups by reacting with acetylcoenzyme A. It might then be largely incidental if the resulting glucuronide were less toxic or active physiologically than the parent acceptor. Ester sulphate formation might conceivably be regarded in the same light, for many hydroxyl compounds form both glucuronides and ester sulphates, and in liver slices the two processes apparently compete for the available phenol acceptor (Storey, 1950). Evidence for an active form of sulphate which will react directly with the hydroxyl compound has been presented by Bernstein & McGilvery (1952). Boström & Månsson (1953) showed that the uptake of $^{35}SO_4$ into the chondroitin sulphuric acid of cartilage slices was stimulated by a thermostable substance present in calf liver, and they suggested that it might be a cofactor for the sulphate-esterifying system. All these observations encourage the hope that further study of the sulphate-conjugation mechanism and of the reactions undergone by UDPglucuronic acid may yield considerable insight into the manner of mucopolysaccharide synthesis.

SUMMARY

1. The active liver factor responsible for glucuronide synthesis in liver suspensions has been isolated from liver extracts by ethanol fractionation of barium salts and ion-exchange chromatography, and shown to be a nucleotide containing uridylic acid, total P, labile P and glucuronic acid in the molecular proportions 1:2:1:1.

2. Hydrolysis of the nucleotide with N acid for 15 min. at 100° gave uridine 5′-phosphate, inorganic P and glucuronic acid.

3. Hydrolysis with 0·1N acid for 7 min. at 100° gave uridine 5′-pyrophosphate, uridine 5′-phosphate, glucuronic acid 1-phosphate, glucuronic acid and a trace of inorganic phosphate.

4. On the basis of the above findings it is proposed that in the nucleotide the uridine 5′-phosphate is linked to the glucuronic acid 1-phosphate through a pyrophosphate bond. It is suggested that the nucleotide be termed 'uridine-diphosphate-glucuronic acid' (UDPglucuronic acid).

5. Confirmation of this structure has been obtained from the study of the action of Russell's viper and cobra venoms.

6. The available evidence suggests that the reaction between the UDPglucuronic acid and the acceptor (ROH) is

UDPglucuronic acid + ROH →
UDP + R–O—glucuronic acid.

7. The possible relationship of this reaction to mucopolysaccharide synthesis is discussed.

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Metabolism of Thyroid Hormones

THE DEIODINATION OF THYROXINE AND TRIIODOTHYRONINE *IN VITRO*

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Recent work on the occurrence and properties of 3:5:3′-triiodo-L-thyronine (Gross & Pitt-Rivers, 1952*a*, *b*, *c*; Roche, Lissitzky & Michel, 1952) strongly suggests that this substance is the active form of the thyroid hormone. On the other hand, it is well established that the main thyroid substance circulating in the blood is thyroxine (Harington, 1944; Taurog & Chaikoff, 1948; Laidlaw, 1949; Leblond & Gross, 1949). It therefore seems necessary to postulate the conversion of thyroxine into triiodothyronine in the peripheral tissues, a process which has not been demonstrated with certainty. Earlier work in this field has recently been reviewed (Maclagan & Wilkinson, 1952; Roche & Michel, 1954) and during the preparation of this paper a more definite claim has been made by

Albright, Larson & Tust (1954), who reported chromatographic evidence for the conversion of thyroxine into triiodothyronine in rat-kidney slices.

One result of this conversion would be the deiodination of thyroxine, and work on this subject is therefore of interest at the present time. There is abundant evidence for such deiodination in whole animals, both intact and thyroidectomized (Elmer, 1938; Gross & Leblond, 1951; Maclagan & Wilkinson, 1954). In contrast to this, no successful experiments on isolated tissues have been reported, although the deiodination of diiodotyrosine in tissue-slice preparations was shown by Hartmann (1950), and similar results with mono- and diiodotyrosine were obtained by Roche, Michel, Michel & Lissitzky (1951). These preparations had no action on thyroxine.

The probable conversion of thyroxine into triiodothyronine was also indicated by our work on antithyroxine compounds such as butyl 4-hydroxy-3:5-diiodobenzoate (BHDB). BHDB inhibits the metabolic effects of thyroxine while augmenting those of triiodothyronine (Maclagan, Sprott & Wilkinson, 1952). It also inhibits the deiodination of both compounds in intact and probably in thyroidectomized animals (Wilkinson, Sprott, Bowden & Maclagan, 1954; Wilkinson & Feetham, 1954). These results are readily explained if we assume that BHDB acts by interference with the conversion of thyroxine into triiodothyronine and also with the destruction of triiodothyronine in the body, a process presumably involving further deiodination.

Further evidence was sought in a study of the deiodination of thyroxine by *in vitro* methods, and the present communication presents fresh facts on this subject. A preliminary account has appeared elsewhere (Maclagan & Sprott, 1954).

MATERIALS AND METHODS

Tissues. The tissues were obtained from male albino rats weighing 200–300 g. The animals were killed by a blow on the head and the required organs removed without delay, placed in ice-cold 1·15% (w/v) KCl (10 ml./g. tissue) and comminuted in an 'Atomix' homogenizer for 1 min. The homogenates were filtered through cotton wool before use.

Substrates. Sodium L-thyroxine and sodium triiodo-L-thyronine were supplied by Glaxo Laboratories Ltd., and radioactive L-thyroxine, labelled in positions 3′ and 5′ with ^{131}I, was purchased from the Radiochemical Centre, Amersham. It had an initial specific radioactivity of 1·05 μc/μg. Non-radioactive thyroxine and triiodothyronine were 0·1% (w/v) solutions in a medium containing 0·0067 M-Na_2CO_3 and 0·06 M-Na_2HPO_4. The solution of radioactive thyroxine, in 50% (v/v) propylene glycol (propane-1:2-diol) at pH 8, was diluted with distilled water to yield a solution containing 4 μg. substrate/ml.

The purity of substrates was checked by paper chromatography using butanol–dioxan–ammonia (Gross, Leblond, Franklin & Quastel, 1950), the chromatograms being either treated with the ceric sulphate reagent of Bowden & Maclagan (1954) or examined by autoradiography.

Buffers. The buffer solutions employed in this work were 0·067 M sodium phosphate, 0·1 M glycine in 0·1 M-NaCl and 0·1 M sodium citrate. In experiments with non-radioactive thyroxine or triiodothyronine ('N' systems), homogenate (10 ml.), buffer (10 ml.) and substrate solution (5 ml.) were incubated at 37° in 50 ml. conical flasks in a Braun rotary Warburg apparatus (Shandon Scientific Co., 6 Cromwell Place, London, S.W. 7). In experiments with labelled thyroxine ('R' systems), homogenate (5 ml.), buffer (5 ml.) and thyroxine solution (1 ml.) were incubated in conical flasks of 25 ml. capacity. The resulting pH of the complete system was measured with a glass electrode (Marconi Instruments Ltd., St Albans, Herts) before incubation.

Measurement of deiodination. After incubation, 4 ml. of 10% (v/v) horse serum were added to each 'R' system followed by 5 ml. of 25% (w/v) trichloroacetic acid (TCA). After standing 5 min. the mixture was filtered through a Whatman no. 50 paper and a suitable sample, usually 2 ml., was diluted to 10 ml. with carrier iodide solution and counted in the liquid Geiger–Müller counter of Veall (1948).

'N' systems were each treated with 10 ml. of 25% TCA and filtered after 5 min. The precipitates were then washed twice with 5% (w/v) TCA (5 ml.). The iodide content of the combined filtrates was estimated by electrometric titration with the Ag/Ag_2S electrode as previously described (Wilkinson *et al.* 1954).

RESULTS

'N' systems

These experiments were conducted only with liver homogenates. Our initial attempts were thought to be negative, because near neutrality the amount of iodide produced was small and was unaffected by preliminary heating to 100° for 30 min. Later, however, it was found that much greater amounts of iodide were formed at acid and alkaline reactions, and that the system could be effectively inhibited by heating for 30 min. in the presence of trichloroacetic acid. The most practical control was, however, to precipitate with trichloroacetic acid and filter immediately after the addition of thyroxine, when only minimal amounts of iodide were found. Fig. 1 shows the results of a typical experiment conducted in this way, from which it will be seen that the deiodination of thyroxine showed two optima at pH 3·5 and 9·5, while in the case of triiodothyronine a single optimum at pH 9·5 was observed. Up to 18% deiodination was recorded in the case of thyroxine, expressed as percentage of total substrate iodine, corresponding to the substantial liberation of 0·6 mg. of iodide. Triiodothyronine was deiodinated somewhat less effectively (up to 7%). It may be noted here that the accuracy of the electrometric titration for iodide is about ± 5 μg.

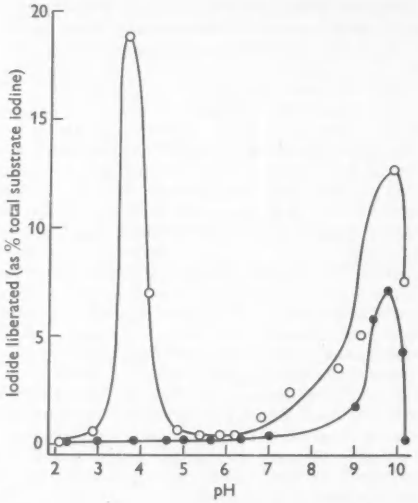

Fig. 1. Effect of pH upon deiodination of L-thyroxine (5 mg.) and triiodo-L-thyronine (5 mg.) by rat-liver homogenates in presence of citrate after 18 hr. ○, L-Thyroxine; ●, triiodo-L-thyronine.

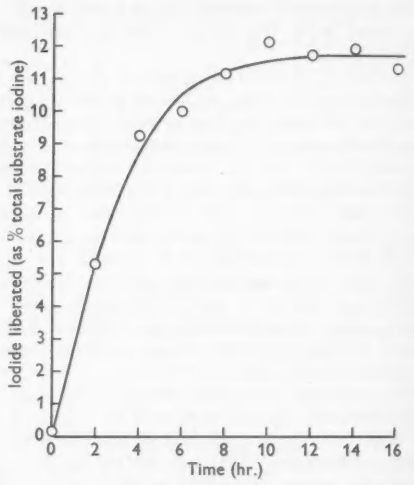

Fig. 2. Rate of deiodination of L-thyroxine (5 mg.) by rat-liver homogenate in glycine buffer at pH 9·3.

Table 1. *Effect of inhibitors on deiodination of* L-*thyroxine and triiodo-*L-*thyronine in 'N' systems*

5 mg. substrate incubated in glycine buffer, for 18 hr. HEHDB, β-hydroxyethyl 4-hydroxy-3:5-diiodobenzoate.

Substrate	pH	Inhibitor	Duplicate estimations of iodide produced (μg.) Without inhibitor	With inhibitor
Na L-thyroxine	9·8	Absence of air (N_2 atmosphere)	376, 445	128, 193
Na L-thyroxine	8·0	KCN, 8×10^{-4}M	170, 141	86, 99
Na L-thyroxine	9·3	BHDB, 10^{-3}M	354, 370	432, 424
Na L-thyroxine	9·3	HEHDB, 10^{-3}M	354, 370	384, 412
Na triiodo-L-thyronine	9·3	BHDB, 10^{-3}M	121, 130	114, 134
Na triiodo-L-thyronine	9·3	HEHDB, 10^{-3}M	121, 130	134, 120

Table 2. *Deiodination of* ^{131}I-*labelled* L-*thyroxine in various buffers*

4 μg. substrate incubated for 3 hr. Results expressed as percentage of substrate radioactivity. Control system with phosphate buffer at pH 6·1 showed 8% deiodination at zero time.

pH	Buffer: Phosphate	Citrate	Phosphate + citrate	Glycine	Glycine + citrate
4·4	15	8	8	—	—
5·6	84	10	10	—	—
6·1	79	9	9	—	—
6·9	62	—	9	—	—
7·8	32	—	10	—	—
8·2	—	—	—	40	10
9·1	—	—	—	9	9

The finding of optimum deiodination at these two unphysiological pH levels suggests that the results may have been partly dependent on the limited solubility of both thyroxine and triiodothyronine in the neutral range. The amount of deiodination at physiological pH was in fact too small for accurate study by the method used.

The reaction is a fairly rapid one, as shown in Fig. 2. This experiment which was conducted at pH 9·3, shows appreciable deiodination after 2 hr., and maximal results after 8 hr.

The deiodination was considerably reduced by exclusion of air and by the presence of 8×10^{-4} M-KCN, but was not affected by antithyroxine substances (Table 1). While no direct comparison between citrate and other buffers was made, the results do not suggest that citrate was an inhibitor at pH 9·5 (cf. Fig. 1 and Table 1).

'R' systems

Deiodination by 'R' systems with rat-liver homogenate was found to be strongly inhibited by citrate, a factor which invalidated many of our earlier experiments. This is shown in Table 2, from which it will be seen that 0·1 M sodium citrate inhibits almost completely at all reactions between pH 4·4 and 8·3. At higher pH values (9·15) the deiodination was minimal and approached the control value.

In phosphate buffer the 'R' system showed a broad pH optimum between pH 5 and 7 (Fig. 3). It will be seen that considerably greater percentage deiodination was observed in 'R' systems (up to 72%) than in 'N' systems. However, the results are not directly comparable, since the radioactive method measures only the liberation of the 3′ and 5′ iodine atoms, the results being expressed as percentage of substrate radioactivity. Furthermore,

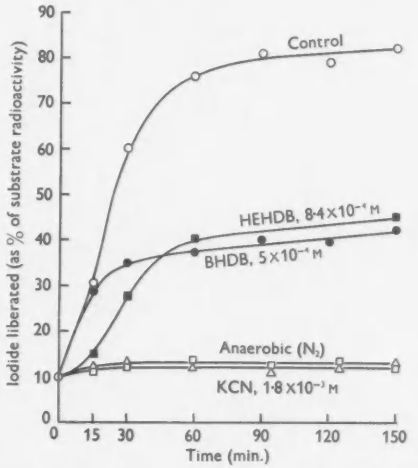

Fig. 4. Effect of inhibitors on rate of deiodination of ^{131}I-labelled L-thyroxine (4 μg.) by rat-liver homogenate in phosphate buffer at pH 6·0.

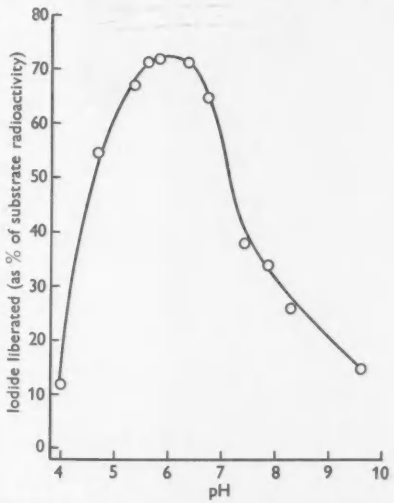

Fig. 3. Effect of pH upon deiodination of ^{131}I-labelled L-thyroxine (4 μg.) by rat-liver homogenate in phosphate buffer at pH 6 in 3 hr.

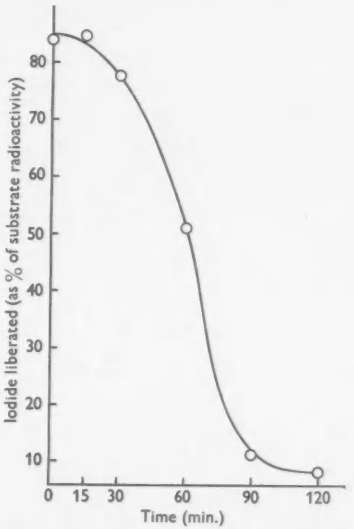

Fig. 5. Effect of heating at 100° on deiodination of ^{131}I-labelled L-thyroxine (4 μg.) by rat-liver homogenate in phosphate buffer at pH 6 (3 hr. incubation).

the concentration of substrate in the 'R' systems is lower by a factor of approximately 1000.

Fig. 4 shows the effect of inhibitors on the rate of deiodination. It may be noted that since the thyroxine was labelled only in the 3′ and 5′ positions, any deiodination in excess of 50% presumably indicates breakdown beyond the triiodothyronine stage.

It will be seen that the system was completely inhibited by exclusion of air or by the presence of cyanide. Two typical antithyroxine compounds BHDB, and β-hydroxyethyl 4-hydroxy-3:5-diiodobenzoate (HEHDB) produced about 50% inhibition of deiodination. This substantial inhibition accords well with our theories of the mode of action of these substances (see Introduction and Discussion).

The 'R' system resembled the 'N' system in its resistance to inactivation by heating. It will be seen from Fig. 5 that very little inactivation occurred at 100° in 30 min., but heating for 2 hr. inactivated the system completely.

Other tissues

The 'R' system was also investigated in homogenates from various other organs with the results shown in Table 3. It will be seen that some deiodination was observed in all tissues studied. Results were maximal with liver and minimal with intestinal mucosa, with the thyroid gland showing only feeble activity.

Table 3. *Deiodination of* ^{131}I*-labelled* L*-thyroxine* (4 μg.) *by rat-tissue homogenates at* pH 6·0

Incubation for 3 hr. in phosphate buffer.

	Radioactive iodide liberated as	
Organ	Counts/min.	Percentage of substrate radioactivity
Liver	27 035	69
	27 395	70
Kidney	17 080	44
	16 550	42
Spleen	15 755	40
	16 390	42
Brain	16 790	43
	16 410	42
Muscle	15 800	40
	14 830	38
Adrenal	14 070	36
Thyroid	9 360	24
Intestinal mucosa	7 210	18
	6 645	17
Control (liver homogenate at zero time)	—	10

Fate of thyroxine residues

Numerous attempts were made to identify other products of the reaction by investigating the trichloroacetic acid precipitates from experiments in which active deiodination had occurred. The thyronine derivatives were extracted with *n*-butanol, sometimes after preliminary solution of the precipitate in a few drops of ammonia (sp.gr. 0·880), and the extract was submitted to paper chromatography. With 'N' systems the chromatograms were developed by the ceric sulphate method but the preponderance of unaltered thyroxine prevented the definite identification of other spots. In the 'R' systems triiodothyronine (18 μg.) was added as carrier before extraction and radioautographs were made. Unfortunately this was done after the main series of experiments at a time when some triiodothyronine had already resulted from spontaneous

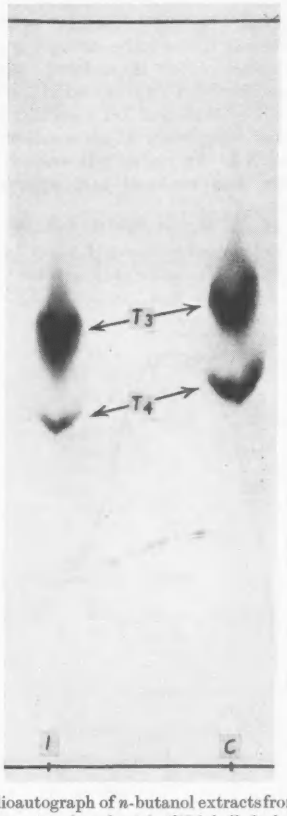

Fig. 6. Radioautograph of *n*-butanol extracts from rat-liver homogenates incubated with ^{131}I-labelled thyroxine in phosphate buffer at pH 6·0 for 2 hr. *C* = control; *I* = incubated; T_4 = thyroxine; T_3 = triiodothyronine.

decomposition of the labelled thyroxine which, as previously noted, does not keep well in solution (Wilkinson *et al.* 1954). The results are shown in Fig. 6, from which it will be seen that incubation has diminished the amounts both of thyroxine and of triiodothyronine present, but the ratio of triiodothyronine to thyroxine appears definitely higher in the incubated extract. The results would be consistent with the conversion of thyroxine into triiodothyronine and the later decomposition of the latter to a non-radioactive product.

DISCUSSION

The experiments recorded appear to establish the existence in the rat of enzyme systems of wide distribution capable of removing iodine from L-thyroxine and from triiodo-L-thyronine.

The evidence suggests the presence of two different aerobic heat-resistant systems which we have called for convenience 'R' and 'N'. System 'R', which was demonstrated with labelled thyroxine (4 μg. per system) had optimum activity at about pH 6 and was inhibited by citrate and by antithyroxine compounds. System 'N', demonstrated with higher concentrations of unlabelled thyroxine (5 mg. per system), had two regions of optimum activity at pH 3·5 and 9·5 and was not inhibited by citrate or by antithyroxine compounds. This system also attacked triiodothyronine at pH 9·5.

System 'N' has only been investigated in liver homogenates but system 'R' was found in all organs tested, being most active in the liver. Although the two systems appear to have these important differences it should be borne in mind that they have been investigated, of necessity, at entirely different levels of substrate concentration, so that the distinction may be more apparent than real.

The product of the deiodination of thyroxine has not been established with certainty but it may be triiodothyronine. Since this substance is itself attacked by the same system, the difficulties of demonstrating its presence are obvious. In this connexion it may be noted that the evidence produced by Albright *et al.* (1954) depended purely on the intensity and position of the radioactivity on paper chromatograms. No autoradiographs of these were reproduced and the counting rates given for the triiodothyronine area were of the low order of 5–40 counts/min.

As was expected from our previous work, the 'R' deiodinating system was inhibited by antithyroxine compounds. This inhibition provides further evidence for our theory of the mode of action of these substances, which appears to depend directly upon this inhibition of deiodination (Maclagan *et al.* 1952).

The actual reactions involved in the deiodination are unknown and will need further study. The aerobic nature of the process and the inhibition by cyanide indicate that oxidative processes are involved, a conclusion which has to be reconciled with an overall reaction which is presumably one of reduction. Further unusual features are the marked inhibition by citrate and the heat-resistant nature of the system, which requires 2 hr. at 100° for complete inactivation.

The ready deiodination observed under the condition of our experiments is somewhat surprising in view of the many previous unsuccessful attempts to demonstrate the process both by ourselves and by others. In our own work the principal stumbling blocks appear to have been the very limited solubility of thyroxine in the neutral range, the heat-resistant nature of the system, and the inhibition by citrate. Thus, in the non-radioactive experiments convincingly positive results could only be obtained at reactions sufficiently acid or alkaline to dissolve the thyroxine. In many of the earlier experiments also the 'controls' were heated for only 30 min., when they showed no difference from the unheated incubated systems, such results being regarded as negative. The use of citrate buffers also probably accounted for many negative results.

It is hoped that the further study of these deiodinating systems will help to throw light upon the peripheral action of the thyroid hormone.

SUMMARY

1. Two aerobic partially heat-resistant systems which deiodinate L-thyroxine and triiodo-L-thyronine have been demonstrated in homogenates from the liver and other tissues of the rat.

2. 'N' systems, demonstrated with non-radioactive thyroxine and triiodothyronine, were effective at pH 3·5 and at pH 9·5. They were inhibited by cyanide and by exclusion of air but not by citrate or by antithyroxine compounds.

3. 'R' systems, demonstrated with ^{131}I-labelled thyroxine, were effective at pH 6 and were inhibited by cyanide, exclusion of air, citrate and antithyroxine compounds.

4. Some evidence was obtained for the conversion of thyroxine into triiodothyronine by 'R' systems.

5. The significance of these results is briefly discussed.

The work was aided by a grant from the Medical Research Council and from the Central Research Fund of the University of London to one of us (N.F.M.) and by grants from the Governors' Discretionary Fund of Westminster Hospital, which are gratefully acknowledged. We are also indebted to Mr A. J. Feetham and to Mr C. H. Bowden for valuable help, and to Miss Ann Burgess for technical assistance. The thyroxine and triiodothyronine were generously supplied by Glaxo Laboratories Ltd.

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Separation of Oestrogens in Urinary Extracts by Partition Chromatography

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In a method developed for the quantitative determination of oestrogens in urine (Bauld, 1954*a*) partition chromatography was applied to the separation and purification of oestrone and oestradiol-17β. Selection of a suitable solvent system was based on the following considerations. The desirability of a system with the oestrogens partitioning markedly in favour of the stationary phase is obvious from a rearrangement of the equation of Butt, Morris, Morris & Williams (1951) to express retention volume in terms of the other factors. In addition, for a routine method it is preferable to have the oestrogens freely soluble in one or other of the phases. Investigation of the contaminants of the oestrone–oestradiol-17β fraction of male urinary extracts (Bauld, 1954*a*) showed the presence of acidic and basic material, and this made it desirable to have aqueous alkali as the stationary phase. Carol, Haenni & Banes (1950) reported the use of an aqueous sodium hydroxide–benzene system for the separation of the oestradiol epimers, and this system was adopted, since it met the requirements noted above, even though it was considered probable that a non-linear isotherm would result.

The ease of separation of oestriol from oestrone and oestradiol-17β by partition between water and benzene (cf. Bachman & Pettit, 1941) rendered the use of chromatography unnecessary for this purpose. Oestriol was removed from the benzene solution of phenols and neutrals with water (Bauld, 1954*a*) and separated from the hydrophilic contaminants in this fraction by a partition chromatogram with 70% methanol–ethylene dichloride, a solvent system discovered empirically.

The factors influencing the elution pattern of both chromatograms were investigated in detail.

METHODS

Purification of materials

Reagents used were A.R. unless otherwise stated. The final distillation of solvents was done in all-glass apparatus.

Benzene. This was refluxed for 30 min. with H_2SO_4 (50 ml./l.), washed with N-NaOH (B.P.) (4 × 0·1 vol.), water (to neutrality), dried over anhydrous Na_2SO_4, and distilled.

Ethylene dichloride. This reagent (I.C.I., metal or stoneware containers) was washed with N-NaOH (B.P.) (3 × 0·1 vol.), water (to neutrality), dried over anhydrous Na_2SO_4, distilled, and redistilled within 24 hr. of use.

Methanol (I.C.I. refined). This was refluxed for 16–20 hr. with NaOH pellets (5%, w/v) and Zn dust (5%, w/v) and distilled twice.

Celite 535 (Johns Manville and Co. Ltd., London). This was partially calcined at 400° for 4 hr., stirred with an excess of HCl and allowed to stand overnight. The solid was washed with water until the washings were free of Cl^- ($AgNO_3$ test), Fe^{3+} (SCN^- test), and the pH was that of distilled water (glass electrode). The material was then dried at 110° for 48 hr., cooled in a vacuum desiccator and stored in containers with tightly fitting glass stoppers.

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Apparatus

The apparatus used for chromatography is illustrated in Fig. 1. Fractions were collected in tubes graduated at 2 ml.

Preparation of chromatograms

Equal volumes of the aqueous and organic solvents, at the temperature at which the chromatogram was to be run, were shaken together (1–2 min.) and allowed to stand until clear. The separated aqueous (stationary) phase was added in known volume to dry Celite in a beaker and stirred for 5 min. An excess of the organic (mobile) phase was then added to the damp Celite; a uniform slurry, obtained by thorough mixing, was added in small portions (enough to give 1–2 cm. of height of column) to an empty chromatogram tube supported with the open end below the surface of a few ml. of mobile phase in a test tube. The plunger (Fig. 1) was moved rapidly up and down the column below the surface of the solvent through the slurry until uniform particles were obtained. These were then packed in small segments (about 2 mm.) by a slow firm movement of the plunger which trapped some of the particles and compressed them into a pad. The most suitable size of hole in the plunger depended upon the interfacial tension of the slurry (cf. Martin, 1949) and holes of standard wire gauge 19 and 22 (1·02 and 0·71 mm.) were used for the NaOH–benzene and 70% methanol–ethylene dichloride systems respectively.

The process described above was repeated until the required height of column was obtained. Sufficient pressure was exerted during packing to give a rate of flow of solvent (hereafter called the percolation rate) of 10–12 ml./hr. Before use, the percolation rate was measured by allowing 5 ml. of mobile phase to pass through the column under gravity. The top surface of the column was always covered with mobile phase except momentarily before the application of the solutes. For routine assay an excess of slurry was prepared and the chromatogram packed to a definite length.

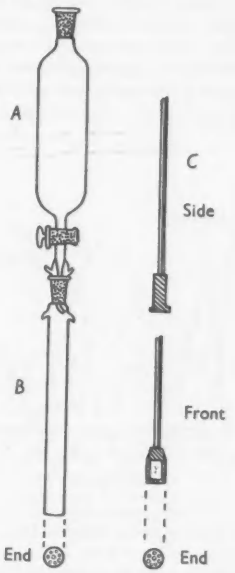

Fig. 1. *A*. Solvent reservoir with capillary outlet and B 19 cone-joint. *B*. Chromatogram column (1 × 15 cm.) with B 19 socket-joint. *C*. Plunger for packing chromatograms; the disk of the plunger should fit the column *B* closely.

Application of solutes and elution

The substances to be separated were dissolved in 1 ml. of mobile phase, transferred to the top surface of the chromatogram, allowed to enter the column completely, and the procedure was repeated twice with 1 ml. washings of the original container. Mobile phase was then added and collection of the eluate begun. The effluent appearing during the application of the solutes is not included in the volume of eluate shown in this report, but must be added when retention volumes are being determined.

Colorimetric estimation

Solvent was removed from the chromatogram eluates by heating in a boiling-water bath in a current of air and the oestrogen content of the dry residue determined by the method already described (Bauld, 1954*b*), the colour correction being applied except in the case of oestrogen-free residues as indicated in the Results section.

Preparation of urine residues

The 'oestriol' and 'oestrone–oestradiol-17β' fractions of hydrolysed urine were prepared as described (Bauld, 1954*a*).

Determination of column parameters and partition coefficients

For these determinations a slurry containing a definite weight of Celite was packed in the column and the length of the chromatogram measured. The cross-sectional area of the inert phase (A_I) was determined from the weight of dry Celite used, its density (2·34), and the length of the column (L) (cf. Martin & Synge, 1941). The cross-sectional area of mobile phase (A_L) was determined from L and the retention volume of a dye with zero partition coefficient for the stationary phase (cf. Butt *et al.* 1951). The cross-sectional area of the stationary phase (A_S) was calculated by the difference between the cross-sectional area of the empty chromatogram and the sum of A_I and A_L. The value should agree within the limits of experimental error with the theoretical value calculated from the volume of stationary phase added to the Celite; if significantly lower, stationary phase is being lost from the chromatogram. The partition coefficient was calculated from the equation of Butt *et al.* (1951).

RESULTS

Separation and purification of oestrone and oestradiol-17β

Fig. 2 shows the elution pattern of oestrone and oestradiol-17β from a chromatogram with N-NaOH as the stationary and benzene as the mobile phase. Also shown is the eluate from a similar

chromatogram to which was applied the 'oestrone, oestradiol-17β, neutral' fraction of male urine. In the second case a brown zone (1 cm. wide) was left on top of the column even after collection of 150 ml. of eluate. Excellent purification and separation were achieved, but the great delay in elution of oestradiol-17β was undesirable for routine determinations. Accordingly, after collection of the eluate from 10–50 ml. (oestrone fraction) the eluent was changed to unequilibrated ethylene dichloride–benzene (3:1, by vol.). This procedure was used for a year; in all the chromatograms carried out during this time oestrone was eluted by 40 ml., and subsequently the solvent change was made at this point. Fig. 3 shows the results of chromatograms of oestrone and oestradiol-17β, and the corresponding fraction of male urine, under these conditions.

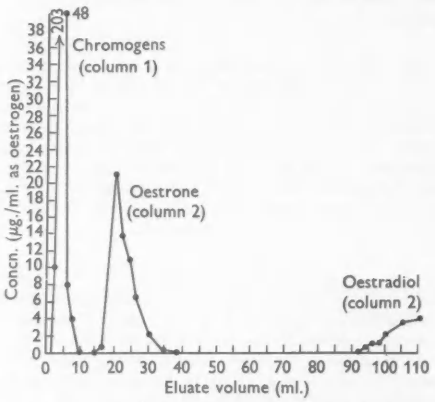

Fig. 2. Chromatography of chromogens of 'oestrone–oestradiol-17β, neutral fraction' of male urine (on one column) and of pure oestrone and oestradiol-17β (on a second column). The characteristics of both columns were: 1 × 13 cm.; mobile phase benzene, stationary phase 0·8N-NaOH; 0·9 ml. stationary phase/g. Celite; percolation rate 9–10 ml./hr. The concentration of male urine chromogens is expressed as oestrone and is calculated from uncorrected colorimetric readings.

Investigation of variables affecting the elution pattern

Normality of stationary phase. The elution of 150 μg. of oestrone and oestradiol-17β from chromatograms with different concentrations of aqueous NaOH is shown in Fig. 4. The retention volumes varied when the stationary phase was less than 0·7N. Increase in concentration to 1·0N gave the same elution pattern as was shown with 0·7N and 0·8N-NaOH. The stationary phase adopted was 0·8N-NaOH.

Temperature. Preliminary experiments indicated that the effects of temperature and of concentration of the stationary phase were interrelated. Fig. 5 shows the results of experiments in which both these factors were varied. With 0·1N-NaOH no separation was obtained at 21°, both oestrogens being eluted by 8 ml., whereas at 16° oestrone and oestradiol-17β were just separable. With 0·8N-NaOH variation in temperature still had an effect on elution patterns but much less markedly. It

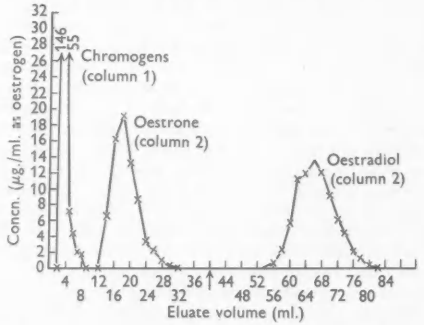

Fig. 3. Chromatography of chromogens of 'oestrone, oestradiol-17β, neutral fraction' of male urine (on one column), and of pure oestrone and oestradiol-17β (on a second column). The characteristics of both columns were: 1 × 12 cm.; mobile phase benzene, stationary phase 0·8N-NaOH; 0·8 ml. stationary phase/g. Celite; percolation rate 10 ml./hr. The eluent was changed at ↑ to ethylene dichloride–benzene (3:1, by vol.). The concentration of male urine chromogens is expressed as oestrone and is calculated from uncorrected colorimetric readings.

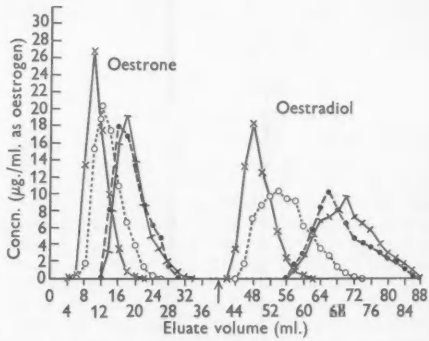

Fig. 4. Effect of alkali concentration of stationary phase on elution of oestrone and oestradiol-17β. The characteristics of the four columns were: 1 × 15 cm.; mobile phase benzene, stationary phase aqueous NaOH of the concentrations shown, 0·9 ml. of stationary phase/g. of Celite; temp. 21 ± 0·5°; percolation rate 9–10 ml./hr. The eluent was changed at ↑ to ethylene dichloride–benzene (3:1, by vol.). ×—×, 0·5N-NaOH; ○---○, 0·6N-NaOH; ●– – –●, 0·7N-NaOH; ——, 0·8N-NaOH.

was apparent that with a 12 cm. column, the temperature should be kept below 20° to allow an adequate zero elution zone between oestrone and the fast-running chromogens. The partition coefficient of oestrone between 0·8 N-NaOH and benzene decreases by 35 % as the temperature is raised from 18 to 37°.

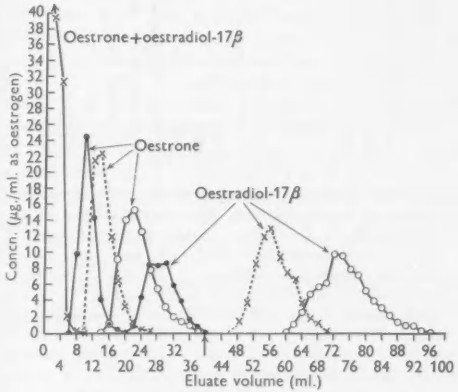

Fig. 5. Effects of alkali concentration of stationary phase, and of temperature, on elution of oestrone and oestradiol-17β. The characteristics of the four columns were: 1 × 12 cm.; mobile phase benzene, stationary phase aqueous NaOH of the concentrations shown; 0·8 ml. of stationary phase/g. of Celite; temp. as shown; percolation rate 9–10 ml./hr. The eluent was changed at ↑ to ethylenedichloride–benzene (3:1, by vol.). ×—×, 0·1 N-NaOH, 21°; ●—●, 0·1 N-NaOH, 16°; ×- - -×, 0·8 N-NaOH, 21°; ○—○, 0·8 N-NaOH, 16°.

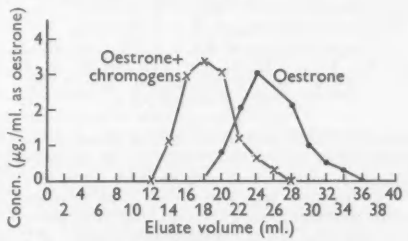

Fig. 6. Chromatography of oestrone (25 μg.) and of oestrone (25 μg.) added to 'oestrone, oestradiol-17β, neutral fraction' of male urine. The same column was used in both cases, the pure oestrone being run first. The characteristics of the column were: mobile phase benzene, stationary phase 0·8 N-NaOH; 0·8 ml. of stationary phase/g. of Celite; temp. 18 ± 0·5°; percolation rate 10 ml./hr. All concentrations were calculated from corr. colorimetric readings. The parameters were: A, 0·9; A_L, 0·36; A_I, 0·18; A_S, 0·36 cm.²; L, 14 cm. (from 6 g. Celite). The partition coefficients were: 4·0 (theoretical); 4·4 (calc. for pure oestrone); 3·2 (calc. for oestrone in presence of male urine chromogens).

Amount of stationary phase added. Investigation of the optimum amount of stationary phase to be added to Celite showed, as expected from theoretical considerations, that the elution of oestrone and oestradiol-17β was delayed as the concentration of stationary phase was increased from 0·6 to 1·0 ml./g. Celite. However, at the higher levels a marked tendency to trailing resulted. The slurry obtained under these latter conditions was visibly different, being granular, whereas that obtained with lesser amounts of stationary phase was flocculent. Initially a concentration of 0·9 ml. of stationary phase/g. Celite was used, and granular slurries with band-spreading were obtained unless rigid precautions were taken to keep the Celite dry (cf. Fig. 4). Use of 0·8 ml./g. of Celite was found to give the most satisfactory results.

Presence of urinary residues. Fig. 6 demonstrates that the retention volume of oestrone is decreased by the presence of the chromogens in the male urine fraction of 'oestrone, oestradiol-17β and neutrals'. The retention volume of oestradiol-17β is similarly affected by chromogens. This important factor has been overcome in the final method by taking 10–30 ml. as the oestrone, 40–90 ml. as the oestradiol, fractions, and as a frequent control procedure collecting the eluate from 8 to 10 and 30 to 40 ml. to demonstrate the absence of oestrogen. In the column shown, A_S and A_L were found to be equal (0·36 cm.²), with the calculated partition coefficient agreeing with the theoretical for pure oestrone but lower in the presence of the urinary contaminants.

Other variables. Oestrogens were eluted somewhat faster from columns packed to give a percolation rate of 20 ml./hr., with gravity feed. The calculated cross-sectional areas of such chromatograms showed the decreased retention volumes to be explainable on the basis of increased A_L/A_S resulting from the less firm packing. The percolation rate of 10–12 ml./hr. was selected for ease of reproducibility.

Figs. 2–6 demonstrate the tendency to trailing boundaries seen in this system. The partition coefficient of oestrone measured at concentrations of 10 and 1 mg./ml. shows a decrease (in favour of the aqueous alkali) of 25 % at the higher levels.

Purification of oestriol

The chromatogram for the separation of oestriol from urinary contaminants was standardized at the outset. Initially the temperature was maintained at 25 ± 0·5° in a thermostatically controlled air enclosure. A series of experiments was conducted at intervals over a period of a year and the uniformity of elution patterns from 1 × 10 cm. columns is demonstrated in the scatter diagram (Fig. 7). For convenience in the final method the chromatograms are run in a room kept at 18 ± 1°. Fig. 8 shows a

typical separation of oestriol from urinary contaminants, and also the elution pattern of pure oestriol under the same conditions. The close agreement between the calculated and theoretical values for partition coefficient is shown.

DISCUSSION

The elution of oestrone and oestradiol-17β described in this report differs from that found with the same solvent system by Swyer and his co-workers (Swyer & Braunsberg, 1951; Braunsberg, Stern & Swyer, 1952), in that their retention volumes are smaller and all three oestrogens are separated on the same column. Since they do not specify the height of their column, calculation of the cross-sectional area of the phases is not possible.

Their method is quick, but the zero elution zone between oestrone and oestradiol-17β is small and oestriol is applied to the chromatogram at a time when its characteristics are markedly changing. One would expect purification to be poor, and this was demonstrated by Bitman & Sykes (1953). The latter investigators displace oestriol by passing carbon dioxide up through the column, a process which must cause gross distortion of the chromatographic zones. Moreover, the method of transferring oestriol in benzene (Bitman & Sykes, 1953) is unlikely to be quantitative.

The variation of elution pattern with alkali concentration of the stationary phase was to be expected from the known variation of partition coefficient under these circumstances (cf. Bachman & Pettit, 1941; Mather, 1942). The marked variation with temperature suggests that the solubility of alkali in benzene plays an important part in determining the distribution of the oestrogens. In instances where it is more convenient to control the temperature at higher levels, the separation between the zones can be maintained by increasing the column length.

The increase in retention volume of the oestrogens as the ratio of stationary to mobile phase increases is in accord with theoretical considerations. The irregular pattern obtained when 1·0 ml. of stationary phase/g. of Celite was used may have been due to the resultant increase in particle size. This would increase the time necessary for equilibration, and as pointed out by Glueckauf (1949) cause a tendency to channelling down the walls of the column. The percolation rate adopted in this investigation was 12–13 cm./hr. Howard & Martin (1950) used 18–44 cm./hr., but stated that separations were better with flow rates down to 10 % of this. In the columns used, percolation rates of 10 ml./hr. were readily obtained without pressure by firm packing of the chromatogram. The conformity of the retention volume with theoretical values suggests that under

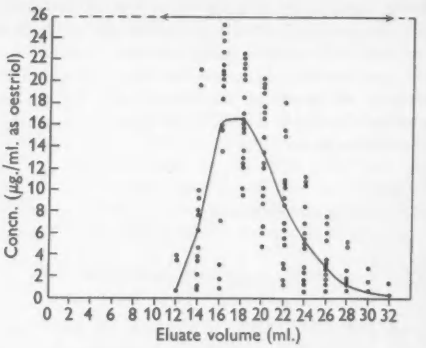

Fig. 7. Scatter diagram showing elution of oestriol from successive chromatograms. The characteristics of the columns were: 1 × 10 cm.; mobile phase ethylene dichloride, stationary phase 70% (v/v) methanol; 1·0 ml. stationary phase/g. of Celite; temp. 25 ± 0·1°; percolation rate 10–12 ml./hr. The volume from 10–32 ml. was taken as the oestriol fraction in the routine analyses during this period.

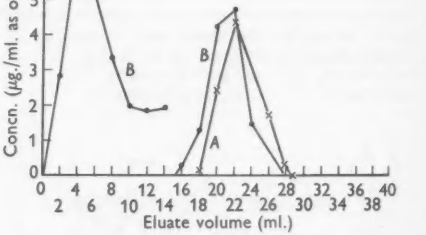

Fig. 8. Chromatography (*A*) of oestriol (25 μg.) and (*B*) of oestriol (25 μg.) added to 'oestriol fraction' of male urine. The same column was used in both cases, the pure oestriol being run first. The column characteristics were: mobile phase ethylene dichloride, stationary phase 70% (v/v) methanol; 1·0 ml. of stationary phase/g. of Celite; temp. 18 ± 0·5°; percolation rate 10 ml./hr. The concn. of eluate from 0 to 14 ml. was calculated from uncorrected colorimetric readings. The parameters were: *A*, 0·9; A_L, 0·41; A_I, 0·17; A_S, 0·32 cm.^{2}; *L*, 10 cm. (from 4 g. Celite). The partition coefficients were: 6·3 (theoretical); 6·5 (calc. for pure oestriol and oestriol in the presence of male urine chromogens). The break in *B* (oestriol + chromogens) is due to the fact that the colorimetric determinations of the eluate residues from 0–14 ml. (which are zero in terms of the colour correction) are shown as the uncorrected values to indicate the amount of non-specific oestrogen; the values from 16 ml. onwards are corrected.

these conditions equilibration is being reached. Slight variations between different workers in the technique of packing are inevitable. The important considerations are: uniformity of the chromatogram, demonstrated by the absence of channelling in dye studies, and obtained by exerting uniform pressure over the whole surface of the chromatogram as the segments are built up; maintenance of the ratio A_L/A_S at approximately unity, demonstrated by dye studies, and obtained by exerting very firm pressure during packing; reproducibility in the packing procedure, demonstrated by uniformity in the retention volume of the dye, and obtained by consistency in the amount of pressure exerted during packing. Once these have been standardized in a preliminary group of chromatograms packed for dye studies, control in routine analysis is obtained by checking the percolation rate and relation between weight of Celite in slurry and column length.

In order to ensure quantitative transfer of the residues to the chromatogram, the flask is washed 3 times with relatively large volumes of mobile phase. Since most of the solutes dissolve in the first wash, this is allowed to enter the chromatogram separately in order to minimize frontal analysis in the bulk of the solutes. The observed variation of partition with concentration is the probable cause of the trailing boundaries seen in the alkaline chromatograms (cf. Levi, 1949).

No investigation was conducted into the most suitable column dimensions. In elution analysis the concentration of solutes on the columns must be kept low, and in view of the large amounts of urinary contaminants a relatively large column was used. Dr S. M. Partridge (personal communication) recommended maintaining a height/diameter ratio of approximately 10/1. Glueckauf (1949) pointed out that low ratios lead to irregular packing and tilting of boundaries, while high ratios cause irregular elution owing to wall channelling.

The decrease in retention volume of oestrone in the presence of urinary contaminants suggests that these exert the displacement effect to be expected with aqueous alkali as the stationary phase (cf. Martin, 1949). The elution of oestriol is unaffected by the presence of urinary contaminants indicating a pure elution analysis.

With the variety of applications of the countercurrent principle now available for the separation of oestrogens in urine extracts, it is desirable to discuss the relative merits of the different methods. Stimmel's (1946) use of alumina was attractive because of the simplicity of operation of adsorption chromatography. The technique has not been widely used owing to the inability of other workers to reproduce his results (cf. Swyer, 1952), an inability probably due to variations in adsorbent (cf. Bates, 1952). Engel (1950) summarized the theoretical and practical limitations of the use of alumina in quantitative methods. Nevertheless, Dr J. B. Brown (to be published), using methylated oestrogens, has produced a satisfactory reproducible separation by adsorption chromatography. The method described by Engel, Slaunwhite, Carter & Nathanson (1950), which uses a countercurrent liquid–liquid extraction train operating discontinuously, is of high precision and dependability, but the complex apparatus and lengthy procedure may prevent its general application to routine analyses. Moreover, oestrogens in urinary extracts are still markedly contaminated (cf. Slaunwhite, Engel, Scott & Ham, 1953). Migeon (1953), by additional distributions, effected further purification but lengthened the procedure.

The method described in the present report is inconvenient in that two partition chromatograms are involved, but the additional labour involved appears to be justified by the wide separation between the impurities and the oestrogens. Prof. C. Morris & Dr J. Preedy (personal communication) have separated the three oestrogens on one chromatogram by gradient elution but the method has not yet been applied to urinary extracts.

SUMMARY

1. A method for the separation and purification of oestriol, oestrone and oestradiol-17β in extracts of hydrolysed urine is described.

2. Partition chromatography is used for the purification of oestriol and for the separation and purification of oestrone and oestradiol-17β.

3. The principal factors affecting the elution of oestrogens from the partition chromatograms are described in full.

The author wishes to thank Professor G. F. Marrian, F.R.S., for his supervision of the work; Professor C. J. O. R. Morris for assistance in preparation of the report; and Mr J. Gordon for skilled technical assistance.

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A New Method of Large-Scale Preparation of Hypertensin, With a Note on its Assay

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(*Received* 15 *September* 1954)

The isolation and identification of hypertensin (Braun-Menendez, Fasciolo, Leloir & Munoz, 1939) or angiotonin (Page & Helmer, 1940) has so far been unsuccessful, and this paper describes a new method of preparation designed to produce large quantities of hypertensin for purification.

Hypertensin is formed during the incubation of the enzyme renin, derived from the kidney, with hypertensinogen, a substrate in the globulin fraction of the plasma. Owing to the low yield of hypertensin (probably not more than 1 mg./l.) it must be made on a large scale. Previous workers have reduced the large volumes necessary by salting out the plasma globulins before activation (Braun-Menendez, Fasciolo, Leloir, Munoz & Taquini, 1946). Finally the reaction has been stopped by precipitating the proteins with heat (Edman, 1945) or ethanol (Page & Helmer, 1940), and the filtrate concentrated by vacuum distillation. The renin hypertensinogen reaction is complicated by the concurrent destruction of hypertensin by 'hypertensinase activity' present in both serum and kidney extracts. The serum activity has been destroyed previously by acidification (Braun-Menendez *et al.* 1946), and it is possible to prepare renin with low hypertensinase activity (Pickering & Prinzmetal, 1938).

When starting volumes of 80 l. of blood are used all these described methods are cumbersome, and it has been found that rapid concentration of hypertensin can be achieved by adsorption on to charcoal from the serum and subsequent elution by glacial acetic acid. The hypertensinase activity of the serum does not affect the yield.

MATERIALS AND METHODS

The various types of kieselguhr, Celite 545 and Standard Supercel were supplied by Johns Manville Co. Ltd., Artillery Row, London, S.W. 1.

The charcoal for adsorption was 'animal charcoal technical powder', British Drug Houses Ltd. It was suspended in glacial acetic acid for a few hr., filtered by suction, washed with distilled water and methanol, and dried in an oven at 105°.

For the assay, urethane (British Drug Houses Ltd.), pentapyrollidinium tartrate in polyvidone solution ('Ansolysen Retard' May and Baker Ltd.), (−)-noradrenaline (Bayer Ltd.) and heparin 5000 units/ml. (Evans) were used.

Renin preparation. This was modified from that described by Pickering & Prinzmetal (1938). Rabbit kidney from animals killed 12–24 hr. previously was minced and stirred at 2° with ethanol (2 ml./g. of kidney) for 12 hr. The pulp was gauze-filtered and dried on filter paper at room temperature. This coarse material was powdered and kept its activity indefinitely in the cold (2°). Renin was extracted from the kidney powder by stirring it with 0·9% NaCl (10 ml./g. powder) at 2° for 12 hr. The pulp was filtered through gauze with squeezing and the cloudy filtrate clarified by filtering through a bed of Celite 545 after the addition of Celite 545 to the solution. The renin was precipitated by the addition of 40 g. of $(NH_4)_2SO_4$/100 ml. of filtrate and the precipitate allowed to settle at 2° overnight. The precipitate was filtered through a Whatman no. 50 paper, and the filter paper suspended in distilled water to dissolve the renin. This solution was then ready for use. The optimum amount of renin/l. of serum was discovered by a pilot experiment. (Usually the renin obtained from 150 g. of kidney powder was sufficient to treat 40 l. of serum.) Hypertensinase activity was very low in this renin preparation.

Hypertensin preparation. Stirred ox blood was obtained from the slaughter house in 80 l. batches, and the serum was separated in a no. 5 Alfa Laval milk separator. Frothing was stopped by the addition of a few drops of tributyl citrate or phosphate.

The 40 l. of serum were stirred with an electrically driven stirrer, and powdered animal charcoal was added (7 g./l.). After stirring for 10 min. the charcoal was allowed to settle for 10 min., then the supernatant serum was pumped through the Alfa Laval centrifuge to remove all suspended charcoal. This charcoal was discarded.

The charcoal-treated serum was incubated with renin at room temperature and the temperature of the serum was always close to 20°. More animal charcoal powder was stirred into the serum (7 g./l.), followed by addition of the renin solution. Incubation with stirring was carried on for 30 min., when the charcoal was allowed to settle for 10 min. The serum was pumped through the centrifuge as before, but to facilitate subsequent removal of the charcoal from the centrifuge bowl the centrifuge fins were left out and it was used as an empty bowl. The charcoal in the bowl was then added to that in the bottom of the incubation bin and suspended in 10 l. of distilled water.

Celite 545 filter aid was stirred by hand into the suspended charcoal (300 g. Celite), and it was then filtered through a bed of Standard Supercel filter aid in a large Büchner funnel. Finally it was washed on the filter with 1·5 l. of ethanol. The charcoal was scooped out of the Büchner funnel leaving the filter bed intact. Elution of the hypertensin was carried out by suspending the charcoal cake in 3 l. acetic acid and stirring for 10 min. The acetic acid eluate was then filtered through the same filter bed. After sucking dry, the charcoal was resuspended in 1·5 l. acetic acid and the process was repeated.

The green eluate was then distilled to dryness at about 20 mm. in a 5 l. flask on a boiling-water bath. The residue was taken up in 50, 25, and 25 ml. of distilled water and filtered through a bed of Standard Supercel in a small Büchner funnel. The filtrate was clear yellow and would keep for months at 2° without loss of activity.

Method of assay

The yield was assayed by the pressor response produced in the anaesthetized rat (urethane 100 mg./kg. intraperitoneally). Albino or hooded rats of 250–350 g. were suitable. Occasionally it was necessary to insert a glass tracheal cannula. The blood pressure was lowered by pentapyrollidinium tartrate in polyvidone solution (2·5 mg./100 g. subcutaneously). The blood pressure was measured directly from the carotid artery with a mercury manometer of the type described by Crawford & Outschoorn (1951), and all doses were given into the external jugular vein. A small glass cannula was connected by thin rubber tubing to a 1 ml. tuberculin syringe graduated in 0·01 ml., containing 0·9% saline. This was held by a rubber band on a heavy steel block. Doses were given from a similar tuberculin syringe into the rubber tubing and washed in with 0·15 ml. saline. The dose volume varied from 0·02 to 0·15 ml. Coagulation was prevented by heparin intravenously (200 units/100 g.). Comparisons were made with stock solutions of hypertensin and with (−)-noradrenaline.

RESULTS AND DISCUSSION

It was possible to make large quantities of hypertensin with this method. The yield in terms of (−)-noradrenaline was 0·5–1·0 mg./l. of serum and the dry weight of material in the acetic acid eluate 100–200 mg./l. The basis of this method is a preliminary cleansing treatment of the serum with charcoal, followed by incubation with renin in the presence of more charcoal. Hypertensin is then eluted from the charcoal. While the method was designed for large-scale production, it can be used for volumes of serum as small as 10 ml.

Type of charcoal. The only suitable type was animal charcoal, which was not activated. It was not possible to get full recovery from activated charcoal.

Hypertensinase. Most methods of hypertensin production involve treatment of the serum or purified substrate to destroy 'hypertensinase activity'. This was not found necessary in the present method, the yield was as great with or without such treatment. Hypertensinase activity was usually present in the serum, shown by the greatly decreased amount of hypertensin obtained when the charcoal was added at the end of the period of incubation, instead of at the start. Once adsorbed, hypertensin was immune to destructive factors in the serum, and the charcoal was often left in the serum or in distilled water overnight at room temperature before elution by acetic acid.

Temperature of incubation. The reaction which released hypertensin was shown to be complete at a temperature of 20° within 30 min.

Comparison with other methods. The yield of hypertensin by the present method was always greater than that obtained by three other methods. These methods were: (1) incubation of renin and serum for 10 min. at 37° followed by protein precipitation in an equal volume of boiling distilled water; (2) similar incubation, with protein precipitation in 4 vol. of ethanol (Page & Helmer, 1940); (3) incubation at 2° for 24 hr. within cellophan tubing so that the hypertensin diffused out into surrounding distilled water. In the first two methods previous destruction of the hypertensinase activity in the serum was achieved by acidifying the serum to pH 3·6–4·0 with hydrochloric acid for 20 min. at 25° (Braun-Menendez *et al.* 1946) before return to pH 7·0 with sodium hydroxide. In the last method this was not necessary as hypertensinase is inactive at 2° (Bean, 1942; Sapirstein, Reed & Southard, 1944).

Elution. Glacial acetic acid was always used. Pyridine (20 %, v/v, in water) was partially effective.

Assay. Control of all stages of the manufacture of hypertensin and of the subsequent steps in purification was only achieved by having a reliable method of assay. Previous workers have used the blood-pressure responses in the dog (Braun-Menendez *et al.* 1946) or cat (Edman, 1945); Skeggs, Kahn & Shumway (1951) have also used the rat. An improved method has recently been described in the rat by Dekanski (1954). The present method has the advantage that the test animal is easy to obtain; is sensitive to small amounts of hypertensin, and gives reproducible results over long periods (up to 8 hr.). The blood pressure is usually steady at pressures of 50–80 mm. Hg for 4–8 hr., and there is the further

advantage that under these conditions it is difficult to elicit depressor responses, even when crude extracts are given. A typical comparison of noradrenaline with hypertensin is shown in Fig. 1. While comparisons were made with stock hypertensin, it was of interest to compare it with the most potent pressor substance known.

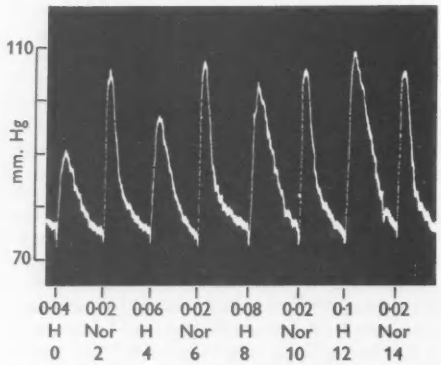

Fig. 1. Comparison of hypertensin with (−)-noradrenaline, showing that 0·02 μg. of noradrenaline is equivalent to between 0·08 and 0·1 ml. of hypertensin. Doses of noradrenaline in μg., of hypertensin in ml. Time in min.

SUMMARY

1. A new method of large scale production of crude hypertensin is described, depending on charcoal adsorption with subsequent elution by glacial acetic acid.

2. A reliable sensitive method of biological assay using the pressor responses in the rat is described.

I would like to express my thanks to Professor G. W. Pickering for continual encouragement, to Mrs P. Brown for technical assistance, and to James Tait, Milfield, Northumberland, for a generous supply of rabbit kidneys.

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The Constitution of the Aliphatic Alcohols in Human Sebum

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Human sebum consists mainly of wax esters, free fatty acids, hydrocarbons and a small amount of glycerides. Whilst the free fatty acids have been investigated in detail (Weitkamp, Smiljanic & Rothman, 1947), little is known about the composition of the other fractions. The unsaponifiable matter has long been known to contain cholesterol (for review of earlier literature, see Čmelik, 1952) and squalene has recently been identified (Sobel, 1949; Mackenna, Wheatley & Wormall, 1952). By chromatographic studies these last authors found 14–20% of aliphatic alcohols in the unsaponifiable matter, but apart from an impure preparation of eicosanol (?) no individual alcohol was isolated.

The present paper describes an investigation into the aliphatic alcohols of human sebum. Sebum (surface skin 'fat') was obtained by extraction of hair from the heads of African (Bantu) males.

METHODS

Reversed-phase partition chromatography of fatty acids. The method of Howard & Martin (1950) (cf. Silk & Hahn, 1954) was extended to effect the resolution of six normal fatty acids ranging in chain length from six to eleven carbon atoms. It was found unnecessary for the present purpose to protect eluate and developing solvent against atmospheric CO_2; also, nitrogen was not purified before entering the titration vessel. The columns were maintained at tap-water temperature (18–22°). For loading of the columns, the acid mixtures were usually dissolved in the developing solvent. It was necessary to prepare a mull only when the mixed acids differed widely in chain length (such as C_5–C_{12}).

The efficiency of separation is illustrated by the elution curve (Fig. 1) for a chromatogram of a synthetic mixture of pentanoic, hexanoic, heptanoic, octanoic, nonanoic, decanoic, hendecanoic and dodecanoic acids (about 7 mg. of each). The C_5 and C_6 acids (unresolved) were eluted with water; C_7 with 10% (v/v) aqueous acetone, C_8 with 25%,

C_9 with 40%, C_{10} with 45%, C_{11} with 50% and C_{12} with 55%. The eluate of this particular chromatogram was collected in 1·8 ml. samples and titrated with 0·02N alkali.

Isolation of total material forming complex with urea. The fraction to be treated with urea was dissolved in 96% (v/v) aqueous ethanol (5–10%, w/v, soln.). An amount of urea was added, equal to about 3 times the anticipated weight of complex-forming material in the fraction. The urea was dissolved by refluxing the solution. After standing overnight at room temperature, the solution was filtered and the precipitate was dried. A second precipitate of urea complex was sometimes obtained by treating the filtrate with more urea, or by further cooling or evaporation to a smaller volume of the filtrate. Complexes were recrystallized from ethanol in which urea (half the weight of the complex) was dissolved. Complexes were decomposed with water, and the alcohol fractions were recovered in ether. Residues which were left in the filtrates were recovered in the same way.

Fractional crystallization of urea complexes. This method was used for resolving mixtures which contained the acetates of a saturated normal, a saturated *iso* and an unsaturated normal alcohol, all having the same number of carbon atoms (cf. Tiedt & Truter, 1952). The prefix '*iso*' denotes a structure containing a terminal *iso*propyl group.

The material (about 10 g.) was dissolved (2%, w/v, soln.) in benzene containing 35% (v/v) of ethanol. Urea (in most cases 2 g.) was added to the solution and dissolved under reflux. After standing overnight at room temperature, the solution was filtered from the precipitated urea complex. The filtrate was again treated with urea (2 g.) to yield a second precipitate of urea complex. This procedure was continued until the precipitate consisted of urea only. The complexes were separately decomposed with water and the acetates recovered.

Chromatography on acid-washed alumina. Alumina (Peter Spence, Widnes, grade H) was washed with hot 2% (w/v) aqueous HNO_3 and then with warm water (distilled) and reactivated at 200°.

The unresolved mixture (about 0·5 g.) of an ω-hydroxy ester and an unhydroxylated ester dissolved in hexane (5 ml.) was passed through a column (12 × 1 cm.) of acid-washed alumina (10 g.) and eluted successively with hexane (35 ml.), hexane containing 10% (v/v) of dry ether (40 ml.) and dry ether (80 ml.). The eluate was collected in 10 ml. fractions which were evaporated and weighed. The unhydroxylated ester was contained in the hexane eluate and the ω-hydroxy ester in the ether eluate.

Isolation of cholesterol through the oxalic acid complex. The method was essentially that of Pickard & Seymour (1945); the detailed procedure was as follows. The cholesterol-containing material was dissolved in anhydrous benzene (20%, w/v, soln.). Anhydrous oxalic acid (10% of the cholesterol-containing material) was added and the solution refluxed for 1 hr. The precipitated complex was filtered off the next day and washed with cold benzene. It was decomposed with ethanolic KOH solution and cholesterol was recovered in ether. The residual material was recovered in the same way.

Infrared absorption spectra. The spectra were recorded with a Perkin Elmer Infrared Spectrometer Model 12C; rock salt prism; path length, 0·5 mm. Alcohols (1·5%, w/v, in CCl_4) were examined in the 7·25 μ. region for the presence of *iso* and normal structures (Freeman, 1952). Unsaturated alcohols (1·5%, w/v, in CS_2) were examined in the 10·35 μ. region for *trans* configurations and in the 10·1 μ. region for terminal double bonds (Freeman, 1953).

EXPERIMENTAL AND RESULTS

Melting points are given corrected. They were measured in capillary tubes immersed in a paraffin bath which was electrically heated at a rate of 1°/6 min.

Extraction of sebum

Hair (28 kg. in batches of about 1·5 kg.) from Africans (Bantu) was obtained from the prison authorities. It was dried at 65° and 20 mm. Hg for 16 hr. and subsequently extracted continuously for 8 hr. with hot commercial 'isoheptane' in a stainless-steel percolator. The extracts were left to stand until the suspended solids settled. The

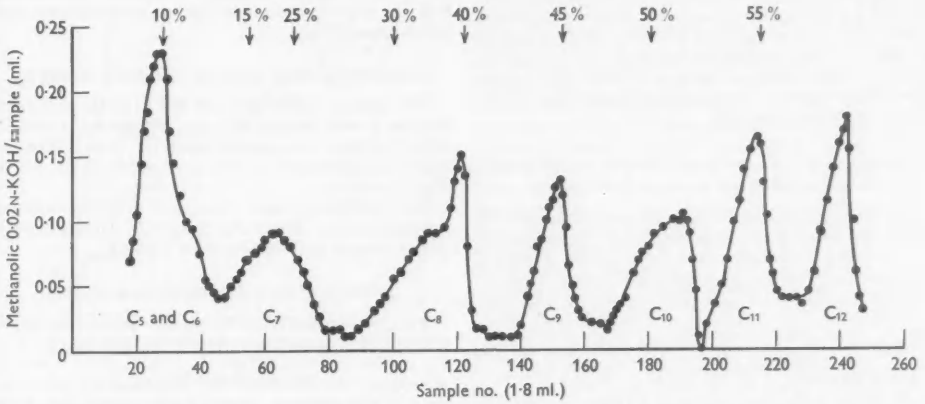


Fig. 1. Resolution of a mixture of pentanoic, hexanoic, heptanoic, octanoic, nonanoic, decanoic, hendecanoic and dodecanoic acids (all *n* acids). Column 75 × 0·8 cm. Hold-up, 23 ml. Changes of solvent (%, v/v, aqueous acetone) are indicated by the arrows.

solutions were siphoned off, the solids washed with 'isoheptane' and the combined solutions evaporated to give crude sebum (1450 g.).

Separation of sebum into free acids, acids released from combination by saponification and unsaponifiable matter

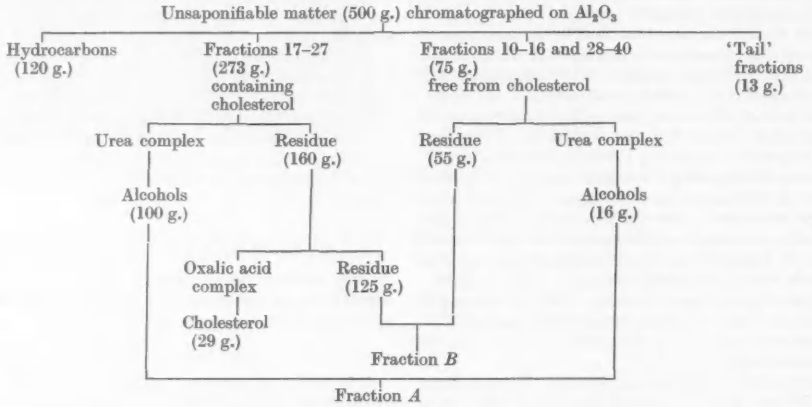
A solution of crude sebum (1335 g.) in hexane (8 l.) was extracted in a separating funnel with 60% aqueous ethanol (4 l.) containing KOH (200 g.). The ethanolic extract, after being continuously extracted with hexane, was acidified (H_2SO_4) and the free acids (458 g.) were obtained by extraction with hexane. Further extraction of the ethanolic solution with ether gave a dark resinous material (24 g.). The combined hexane solutions which contained the neutral material were evaporated to a smaller volume (2 l.). Ethanol (2 l.) containing KOH (170 g.) was added and the mixture stirred for 24 hr. at 40°. Saponification was complete under these conditions. The acids released from combination (285 g.) were separated from the unsaponifiable matter (533 g.) by extraction of the latter with hexane from a solution of the saponified mixture in 60% (v/v) aqueous ethanol. A final continuous extraction (48 hr.) with hexane gave another portion of unsaponifiable matter (14 g.) which was not investigated further.

Separation of hydrocarbons, aliphatic alcohols and cholesterol from the unsaponifiable matter

The non-aromatic hydrocarbons were separated from the unsaponifiable matter by chromatography. Unsaponifiable matter (500 g.) in hexane (10 l.) was passed through a column (35 × 15 cm.) of alumina (Peter Spence, Type H; 5 kg.) and eluted with hexane (12·5 l.), ether (37·5 l.), ether containing 2% (v/v) of 96% (v/v) aqueous ethanol (15 l.), ether containing 10% of 96% ethanol (15 l.), 96% ethanol (10 l.), and finally 96% ethanol containing 10% (v/v) of aqueous 9·5 N-HCl (10 l.). The eluate was collected in 2·5 l. fractions (44 in all), samples from which were evaporated to dryness and examined. The hydrocarbons (120 g.) were contained in the fractions (2–9) which were eluted with hexane and subsequently combined. The 'tail' fractions (41–44; 13 g.) which were eluted with ethanolic HCl were set aside. The remaining fractions were combined into a cholesterol-containing group (17–27; 273 g.) and a cholesterol-free group (10–16 and 28–40; 75 g.).

The cholesterol-containing (273 g.) and the cholesterol-free (75 g.) grouped fractions were separately treated with urea; aliphatic alcohols (100 and 16 g. respectively; subsequently combined and referred to as fraction *A*), and residual fractions (160 and 55 g. respectively) were obtained.

The residual fraction (160 g.) which contained cholesterol was treated with oxalic acid; cholesterol (29 g.) and a residual fraction (125 g.) were obtained. The latter was combined with the cholesterol-free residual fraction above (55 g.). This combined fraction is referred to as fraction *B*. These fractionations are shown in the diagram below:



Acetylation of fractions A and B

Fraction *A* (aliphatic alcohols; 116 g.) was heated with acetic anhydride (350 g.) on a water bath for 4 hr. The excess of solvent was distilled off under vacuum to give the acetates (133 g.). A portion of fraction *B* was similarly converted into acetates (30 g.).

Distillation of the acetylated fractions A and B

Two separate batches (30 and 103 g.) of the acetylated fraction *A* were distilled at 1 mm. Hg through a Piros & Glover micro spinning-band column (cf. Horn & Hougen, 1953). The distillation curve for the larger batch is shown in Fig. 2 (curve *A*).

The acetylated portion of fraction *B* (30 g.) was distilled through the same column (Fig. 2, curve *B*). Only about half of this material distilled over below 240° mm.

Isolation of six saturated normal alcohols

By crystallizations (from methanol, ethanol or acetone, as suitable) of the appropriate distillation fractions (flats C_{14}, C_{16}, C_{18}, and fraction *b* of flat C_{20}; Fig. 2), pure acetates of tetradecan-1-ol, hexadecan-1-ol, octadecan-1-ol, and eicosan-1-ol were obtained. Impure 1-tetracosanyl acetate was obtained by crystallization of fraction *f* of flat C_{24}. Fractional crystallization of urea complexes was applied to fraction *d* of flat C_{22}. The first two complexes obtained were recovered to

yield an impure preparation of 1-docosanyl acetate. The acetates were hydrolysed with ethanolic KOH and the recovered alcohols crystallized to purity (methanol, ethanol, hexane, ether). Analytical data for the alcohols and their acetates are given in Table 1.

Isolation of three saturated iso *alcohols*

Fractions *a*, *c* and *e* (Fig. 2), combined with the corresponding distillation fractions from the smaller batch (30 g.) of acetylated fraction *A*, were separately fractionated by crystallization of urea complexes. Fraction *a* (14 g.) was treated successively with 4 × 10 g. and 4 × 2 g. of urea. The acetate of 18-methylnonadecan-1-ol was recovered from the last (eighth) urea complex; the residue left after urea fractionation contained an unsaturated alcohol, as shown by its high iodine value, but this residue was not used as a source of eicos-10-en-1-ol since a better source (see below) was available. Fractions *c* (10 g.) and *e* (8 g.) were treated with 2 g. lots of urea and divided into 13 (numbered *c* 1–*c* 13) and 11 (numbered *e* 1–*e* 11) urea complexes respectively. Complexes *c* 8 and *e* 8 were decomposed to give the acetates of 20-methylheneicosan-1-ol and 22-methyltricosan-1-ol respectively. The acetates were hydrolysed and the recovered *iso* alcohols crystallized to purity. Analytical data are given in Table 1. The terminal *iso*propyl structure of the three *iso* alcohols was confirmed by their infrared absorption spectra which showed the characteristic splitting of the 7·25 μ. band (Freeman, 1952). The isolated 18-methylnonadecan-1-ol and a synthetic specimen (Horn & Hougen, 1953) had identical infrared spectra.

Isolation and identification of three unsaturated normal alcohols

Eicos-10-*en*-1-*ol*. The single flat which was obtained by distillation of fraction *B* (Fig. 2) contained the acetate of an unsaturated C_{20} alcohol.

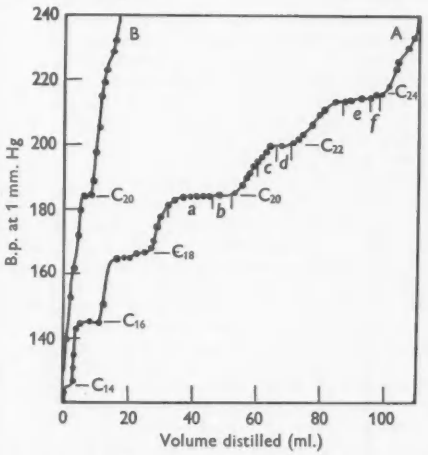


Fig. 2. Distillation curves (for conditions see text): *A*, acetates of the urea complex-forming material of sebum unsaponifiable matter (hydrocarbons excluded); *B*, acetates of the residual unsaponifiable matter.

A sample of the crude acetate was hydrogenated (Pd on $BaSO_4$ in glacial acetic acid) and took up 1·03 mol. prop. of H_2 (based on eicosenyl acetate). The hydrogenated sample melted at 39–41°. The acetate was saponified and the recovered alcohol crystallized from hexane and then methanol to m.p. 64·2–64·6°, unchanged on admixture with authentic eicosan-1-ol. (Found: C, 80·65; H, 14·4. Calc. for $C_{20}H_{42}O$: C, 80·5; H, 14·2%.)

The total fraction of eicosenyl acetate (about 2 g. from 30 g. of acetylated fraction *B*) was saponified, and the recovered alcohol (1·95 g.) crystallized from methanol at −60° to m.p. 23·5–24·4° (analysis, see Table 1). Examination of the infrared spectra revealed that the double bond had the *cis* configuration and that there was no terminal double bond or terminal *iso*propyl group.

The alcohol (1·3 g.) was oxidized with performic acid in acetic anhydride according to the method of Fietelson (1950) for the oxidation of oleyl alcohol. The oxidation product was saponified to give crude trihydroxyeicosane (1·29 g.). Crystallization from hexane–methanol and from ether–methanol gave *trihydroxyeicosane* having m.p. 86·0–86·3°. (Found: C, 72·9; H, 12·9. $C_{20}H_{42}O_3$ requires C, 72·7; H, 12·8%.)

This product, combined with a less pure second crop from the mother liquors (1·0 g.), was oxidized in benzene solution with lead tetra-acetate and a stream of air according to Mendel & Coops (1939; cf. Horn & Hougen, 1953). The recovered acid reaction product (0·28 g.) was esterified with methanol–H_2SO_4 and the mixed esters (0·27 g.) were separated by chromatography on acid-washed alumina into unhydroxylated ester (0·089 g.) and ω-hydroxy ester (0·124 g.).

The unhydroxylated ester was hydrolysed to give the acid (0·080 g.). A portion of this acid (approx. 9 mg.) was examined by reversed-phase partition chromatography, and a single peak was obtained with no traces of neighbouring homologues. A second chromatogram was run with the unhydroxylated acid (4 mg.) in admixture with authentic decanoic acid (4 mg.). The total material was eluted in a single peak and the unhydroxylated acid was therefore decanoic acid.

The ω-hydroxy ester (0·124 g.) was saponified to give the acid (0·116 g.). The acid (in 40%, v/v, aqueous acetone) was passed through the chromatographic column in order to free it from any admixed unhydroxylated acid. The recovered acid was crystallized from hexane–benzene to m.p. 69–69·5° (10-hydroxydecanoic acid has m.p. 75–76° according to Chuit & Hausser, 1929). The acid was dissolved in aqueous alkali and oxidized with 5% (w/v) aqueous $KMnO_4$ at 50°. The oxidation product was worked up in ether, crystallized from water and extracted with boiling hexane to give a product having m.p. 127–129° (raised to m.p. 130–132° on admixture with sebacic acid of m.p. 133°). (Found: C, 59·8; H, 8·4. Calc. for $C_{10}H_{18}O_4$: C, 59·4; H, 9·0%.) Paper chromatograms of C_8, C_9 and C_{10} dibasic acids, of the acid oxidation product and of this last in admixture with authentic sebacic acid, were run according to the method of Long, Quayle & Stedman (1951), except that the papers were sprayed with bromophenol blue and citric acid (Kennedy & Barker, 1951). The C_8 dibasic acid gave R_F, 0·46; C_9 acid, 0·51; C_{10} acid, 0·615; oxidation product, 0·575; a mixture of the oxidation product and C_{10} acid gave a single spot (R_F, 0·585). Although the sebacic acid obtained by the oxidation of the 10-hydroxydecanoic acid was not entirely pure, there can be no doubt about its identity.

Table 1. *Properties of the isolated alcohols and their acetates*

Calc. values for acetyl, C and H percentages are in brackets.

	Acetates				Alcohols					
	M.p. (°)	Acetyl (%)	C (%)	H (%)	M.p. (°)	C (%)	H (%)	X-ray long crystal spacings (Å)		Minimum percentage present (g./100 g. non-saponifiables)
Tetradecan-1-ol	13·4–14·0	17·0	74·6	12·6	37·7–38·0	78·2	14·0	—	39·6	0·2
	14·0[a]	(16·8)	(74·9)	(12·6)	38·26[g]	(78·4)	(14·1)	—	39·7[f]	—
Hexadecan-1-ol	24·3–24·5	15·3	76·1	12·7	49·3–49·4[b]	79·1	14·1	36·8	44·0	1·4
	24·2[a]	(15·1)	(76·0)	(12·8)	49·62[g]	(79·3)	(14·1)	37·4[f]	44·9[f]	—
Octadecan-1-ol	32·7–32·9	13·9	77·05	13·0	58·0[b]	79·9	14·15	41·2	—	2·5
	32·8[a]	(13·75)	(76·9)	(12·9)	57·98[g]	(79·9)	(14·2)	41·3[f]	50·2[f]	—
Eicosan-1-ol	39·7–39·8	13·0	77·5	13·0	65·05	80·4	14·2	45·4	—	3·0
	40±0·5[a]	(12·6)	(77·6)	(13·0)	65·5[g]	(80·5)	(14·2)	—	—	—
Docosan-1-ol	47·6–47·8[d]	—	—	—	70·3–70·4	81·0	14·1	49·8	—	1·3
	49–51[c]	—	—	—	70·6[g]	(81·0)	(14·2)	49·95[f]	—	—
Tetracosan-1-ol	*ca.* 53·5[d]	—	—	—	75·5–75·7	81·3	14·2	53·2	—	2·0
	57[a]	—	—	—	74·8[g]	(81·3)	(14·2)	54·0[f]	—	—
18-Methylnonadecan-1-ol	30·9–32·9[d]	—	—	—	49·2–49·4[b]	80·3	14·2	—	—	0·5
					50·3[e]	(80·5)	(14·2)	—	—	—
20-Methylheneicosan-1-ol	—	—	—	—	55·4–55·9	80·9	14·3	—	—	0·4
					57·1[e]	(80·9)	(14·2)	—	—	—
22-Methyltricosan-1-ol	—	—	—	—	62·4–62·7	81·15	14·3	—	—	0·5
					62·4[e]	(81·3)	(14·2)	—	—	—
Eicos-10-en-1-ol	—	—	—	—	23·5–24·4	81·0	13·7	—	—	0·6
						(81·0)	(13·6)	—	—	—
Docos-12-en-1-ol	—	—	—	—	—	81·4	13·8	—	—	0·4
						(81·4)	(13·7)	—	—	—
Tetracos-14-en-1-ol	—	—	—	—	43·3–43·5	81·6	13·8	—	—	0·5
						(81·7)	(13·7)	—	—	—

[a] Phillips & Mumford (1934).
[b] The m.p. was unchanged by admixture with an authentic sample.
[c] Tiedt & Truter (1952).
[d] The acetate was not obtained pure.
[e] Murray & Schoenfeld (1952).
[f] Malkin (1930).
[g] Ralston (1948).

Docos-12-en-1-ol. As already described, fraction *c* of distillation flat C_{22} (Fig. 2) was divided into thirteen urea complexes. The acetate which was recovered from complex *c*12 was hydrolysed to give an unsaturated C_{22} alcohol (see Table 1). The double bond in this alcohol had the *cis* configuration, as shown by its infrared absorption spectrum, and there was no terminal double bond or terminal *iso*-propyl group.

A sample of the alcohol was hydrogenated and took up 0·88 mol. prop. of hydrogen (based on docosenol). The recovered hydrogenated sample was crystallized from ether to m.p. 70·1–70·2°, unchanged on admixture with authentic *n*-docosanol. (Found: C, 81·0; H, 14·3. Calc. for $C_{22}H_{46}O$: C, 80·9; H, 14·2%.)

A preparation of docosenol (containing also some 20-methylheneicosanol) was obtained from the pooled urea complexes *c*11–*c*13 and the most pentane-soluble part of the recovered alcohols from complexes *c*9 and *c*10. This material (1·55 g.) was oxidized with performic acid and the product saponified to give *trihydroxydocosane* (1·27 g.). Crystallization from ethyl acetate, followed by many extractions with boiling hexane to remove monohydric alcohol, and one crystallization from ether, gave fine prisms, m.p. 87·4–88·1°. (Found: C, 73·9; H, 12·7. $C_{22}H_{46}O_3$ requires C, 73·7; H, 12·9%.)

This product was pooled with a second crop obtained from the mother liquors (total 0·66 g.) and oxidized with lead tetra-acetate and air. The recovered acid reaction product (0·52 g.) was converted into the methyl esters (0·43 g.), and the latter were chromatographed on acid-washed alumina to give unhydroxylated ester (0·15 g.) and ω-hydroxyester (0·2 g.).

The acid (0·093 g.) which was recovered from the unhydroxylated ester was examined by reversed-phase partition chromatography and found to be decanoic acid with no traces of neighbouring homologues. A mixed chromatogram with authentic decanoic acid confirmed this. The acid which was recovered from the first chromatogram was converted into its *p*-bromophenacyl ester, which was crystallized from aqueous ethanol to m.p. 65·1–65·4° (*p*-bromophenacyl decanoate has m.p. 66° according to Hopkin & Williams (1950)). Admixture with authentic *p*-bromophenacyl decanoate gave no depression of the melting point, whereas authentic *p*-bromophenacyl nonanoate depressed the melting point considerably.

The ω-hydroxy ester (0·2 g.) was crystallized from pentane to m.p. 33·1–33·8° (methyl 12-hydroxydodecanoate has m.p. 34–34·5° according to Chuit & Hausser (1929)). Chromatography of the ester on acid-washed alumina followed by crystallization from pentane failed to raise the melting point of the ester. The ester was saponified and the recovered ω-hydroxy acid crystallized (benzene, hexane–ethyl acetate) to m.p. 78·1–81·7° (12-hydroxydodecanoic acid, m.p. 84–85° (Chuit & Hausser, 1929)).

Tetracos-14-en-1-ol. As described before, fraction *e* of distillation flat C_{24} (Fig. 2) was divided into eleven urea complexes. The acetate contained in complex *e*10 was saponified to give an unsaturated C_{24} alcohol. The alcohol was crystallized (methanol, acetone, benzene) to m.p. 43·3–43·5° (see Table 1). The infrared absorption spectrum proved the double bond of the alcohol to have the *cis* configuration, and that no terminal double bond or *iso*-propyl group was present.

A sample of a less pure preparation of the alcohol (m.p. 41·6–42·0°) was hydrogenated and took up 1·06 mol. prop. of hydrogen (based on tetracosenol). The hydrogenated sample was recovered and crystallized from ether to m.p. 75·1–75·3°, unchanged on admixture with authentic tetracosanol. (Found: C, 81·2; H, 14·4. Calc. for $C_{24}H_{50}O$: C, 81·3; H, 14·1%.)

A preparation (1·6 g.) of tetracosenol (containing some 22-methyltricosanol) was obtained from the pooled urea complexes *e*8–*e*11. The material was oxidized with performic acid and the product saponified to give *trihydroxytetracosane* (1·5 g.), crystallized from hexane, ether at −25° and methanol to m.p. 89–90·4°. (Found: C, 74·5; H, 13·1. $C_{24}H_{50}O_3$ requires C, 74·55; H, 13·0%.)

A less pure preparation of trihydroxytetracosane (0·95 g.) was oxidized with lead tetra-acetate and air, and the acid oxidation products (0·56 g.) were converted into the methyl esters (0·5 g.). The esters were chromatographed on acid-washed alumina to give unhydroxylated ester (0·15 g.) and ω-hydroxy ester (0·2 g.).

The unhydroxylated ester was hydrolysed to the acid (65 mg.) and the latter was examined by chromatography on the reversed-phase column. A small fraction of the total material (10–15%) was eluted with 45% acetone and the rest of the material with 50% acetone. The acid contained in the second peak was recovered and converted into its *p*-bromophenacyl ester, m.p. 65·3–65·5° (*p*-bromophenacyl decanoate, m.p. 66°) unchanged on admixture with an authentic specimen of *p*-bromophenacyl decanoate. (Found: Br, 21·5. Calc. for $C_{18}H_{25}O_3Br$: Br, 21·7%.) Confirmation that the unhydroxylated acid fraction was substantially decanoic acid was obtained by a chromatogram of this material admixed with authentic decanoic acid. The smaller fraction of the unhydroxylated acid material which was eluted in front of decanoic acid was recovered and melted at 40°.

The ω-hydroxy ester (0·2 g.) was crystallized from hexane to m.p. 41·7–43·0°. The ester was saponified and the recovered acid crystallized from hexane to m.p. 75–85·5° (14-hydroxytetradecanoic acid, m.p. 91–91·5°; Chuit & Hausser, 1929). (Found: C, 68·85; H, 11·6. Calc. for $C_{14}H_{28}O_3$: C, 68·8; H, 11·55%.) The acid in 45% aqueous acetone was passed through the reversed-phase column, but there was no improvement in the melting point of the acid recovered from the eluate.

DISCUSSION

The sebum was obtained by extraction of hair from a large number of people, and it might therefore have been contaminated with extraneous matter such as hair dressings, though these are not normally accessible to the subjects from whom the hair was obtained. Hair dressings consist mostly of neutral fats (glycerides) and mineral oils (cf. Weitkamp *et al.* 1947), and such materials do not yield a high percentage of long-chain alcohols on saponification. Contamination of the material reported upon here is therefore not likely to be appreciable in amount.

Twelve aliphatic alcohols of human sebum were identified. It is not possible to state with certainty the relative proportion of each present, since intermediate fractions and residues obtained during the lengthy fractionation contain unknown amounts of the various compounds; however, an indication of

the minimum percentages present is given in the last column of Table 1. The alcohols belong to three homologous series, namely: saturated normal alcohols (tetradecan-1-ol, hexadecan-1-ol, octadecan-1-ol, eicosan-1-ol, docosan-1-ol, tetracosan-1-ol), saturated *iso* alcohols (18-methylnonadecan-1-ol, 20-methylheneicosan-1-ol, 22-methyltricosan-1-ol) and unsaturated normal alcohols (eicos-10-en-1-ol, docos-12-en-1-ol, tetracos-14-en-1-ol).

Isolation of the mixed alcohols was effected by precipitation of their urea complexes from unsaponifiable matter that had previously been freed from non-aromatic hydrocarbons. Because the formation of urea complexes was not quantitative, a smaller fraction of alcohols was left in the residue. The mixed alcohols (as acetates) were separated by distillation into six fractions of increasing molecular weight (crude C_{14}, C_{16}, C_{18}, C_{20}, C_{22} and C_{24} alcohols). Each of the three lower-boiling fractions consisted of one saturated normal alcohol only. The three higher-boiling fractions were mixtures of normal, *iso* and unsaturated alcohols, which could not be resolved by distillation. These mixtures were separated by fractional crystallization of their urea complexes. One of the unsaturated alcohols (C_{20}) was present in appreciable amount in the residual unsaponifiable matter which did not form a urea complex, and was isolated from this fraction by distillation of the acetates; iodine values of the distillation fractions indicated that there were unsaturated alcohols present of chain lengths lower than C_{20} and higher than C_{24}, but the quantities were too small for their isolation. Some of these distillation fractions, however, deposited small amounts of crystals which in two of the fractions were most probably anthracene and chrysene (Hougen, 1954).

The saturated normal alcohols were identified by their melting points, elementary compositions, and X-ray long crystal spacings, and in some cases by acetyl values and the melting points and elementary compositions of the acetates. The *iso* alcohols were identified by their melting points, elementary compositions and infrared absorption spectra. There was sufficient material for measurement of the X-ray long crystal spacing of only one of the *iso* alcohols (C_{24}). This alcohol gave no clear X-ray pattern, and an impurity such as tetracosan-1-ol might therefore have been present in the sample. The unsaturated alcohols had one double bond and an unbranched chain as shown by their hydrogenation to the corresponding saturated normal alcohols. The double-bond positions were determined by oxidative cleavage of the alcohols into two acid fragments (ω-hydroxy acids and unhydroxylated acids) followed by the identification of these acids. The possibility was considered that the unsaturated alcohols were mixtures of double-bond position isomers (cf. double-bond position isomers in the free fatty acids of sebum; Weitkamp *et al.* 1947), in which case the acid fission products would be homologous mixtures. The technique of reversed-phase partition chromatography of fatty acids (C_{12}–C_{18} acids (Howard & Martin, 1950); C_{20}–C_{24} acids (Silk & Hahn, 1954)) was extended to include six shorter-chain normal acids (C_6–C_{11}); the unhydroxylated acid degradation products were analysed by this method. The C_{20}, C_{22} and C_{24} unsaturated alcohols each yielded decanoic acid with no traces of its homologues. In one case (degradation product from C_{24} alcohol) a small fraction of unidentified material appeared in the eluate in front of the decanoic acid. This unidentified material was neither a homologue of decanoic acid nor was it 14-hydroxytetradecanoic acid, as was shown by its position in the elution curve and by the melting point of the recovered material. Although this material was not identified, its presence clearly does not influence the argument about the structure of the tetracosenol component. The identification of decanoic acid as a degradation product of the C_{20}, C_{22} and C_{24} alcohols proved their structures to be eicos-10-en-1-ol, docos-12-en-1-ol and tetracos-14-en-1-ol respectively. The corresponding derived ω-hydroxy acids (10-hydroxydecanoic, 12-hydroxydodecanoic and 14-hydroxytetradecanoic acids) proved difficult to purify by crystallization. It was considered sufficient to identify one of them (10-hydroxydecanoic acid) by oxidation to the dibasic acid (sebacic acid).

The saturated normal alcohols of sebum are common constituents of natural waxes. The *iso* alcohols are apparently not of wide occurrence, but have been found in wool wax (Murray & Schoenfeld, 1952). The unsaturated alcohols have not been described before, but somewhat similar alcohols have been found in the seed oil of *Simmondsia chinensis* (eicos-11-enol and docos-13-enol; Green, Hilditch & Stainsby, 1936) and in sperm blubber oil (eicos-9-enol; Toyama, 1938).

In many natural waxes the acids and alcohols are structurally related, and this may suggest a common path in the biosynthesis of these two classes of compounds. Sheep sebaceous secretion (wool wax) is a striking illustration of this phenomenon as the acids and aliphatic alcohols belong to the same four homologous series of normal, *iso*, *ante-iso* and α-hydroxy compounds (Knol, 1954). A similar relationship does not exist between the alcohols and the free fatty acids of human sebum. Acids of odd- as well as even-numbered chain lengths are present, whilst *iso* acids have not been found (Weitkamp *et al.* 1947). The double bonds are differently situated in the acids and the alcohols. The 'combined' acids of sebum have not yet been reported upon in the literature.

SUMMARY

1. Twelve aliphatic alcohols of human sebum have been isolated and identified. They belong to three homologous series as follows: saturated normal alcohols (tetradecan-1-ol, hexadecan-1-ol, octadecan-1-ol, eicosan-1-ol, docosan-1-ol, tetracosan-1-ol), saturated *iso* alchols (18-methylnonadecan-1-ol, 20-methylheneicosan-1-ol, 22-methyltricosan-1-ol) and unsaturated normal alcohols (eicos-10-en-1-ol, docos-12-en-1-ol, tetracos-14-en-1-ol).

2. The reversed-phase partition chromatographic technique for resolution of fatty acid mixtures (Howard & Martin, 1950) has been extended to include six shorter-chain acids having odd and even numbers of carbon atoms.

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The Estimation of Unsaturated Fatty Acids by Reversed-Phase Partition Chromatography

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During an investigation of the metabolism of fat-containing seeds, a method was required for the analysis of milligram quantities of fatty acids. From a number of column-chromatographic methods (Boldingh, 1950; Cropper & Heywood, 1953; Nijkamp, 1953, 1954; Simmons & Quackenbush, 1953), the Howard & Martin (1950) reversed-phase technique was selected for further investigation. This method is effective for the separation of even-numbered straight-chain saturated fatty acids (from C_{12} to C_{18}). It has recently been extended to similar acids from C_{10} to C_{24} (Popják & Tietz, 1954; Silk & Hahn, 1954). Savary & Desnuelle (1953) have investigated the behaviour of oleic and linoleic acids on similar columns and show that these acids are separable. Linoleic acid is eluted rather less rapidly than myristic acid, and oleic acid at the same rate as palmitic acid. In order to separate these saturated and unsaturated acids, Savary and Desnuelle employed ice-cold dilute alkaline permanganate oxidation to convert the unsaturated acids into the corresponding hydroxy acids, which are eluted more rapidly than palmitic acid. The di- and tetra-hydroxy stearic acids are then separated by reversed-phase chromatography on columns of purified castor oil.

We have investigated the behaviour, on Howard & Martin columns, of a large number of unsaturated

fatty acids. This has been combined with the use of the Bertram oxidation method (concentrated alkaline permanganate at room temperature; Markley, 1947) for the removal of the unsaturated from the saturated acids. The treatment degrades unsaturated acids to compounds which are eluted more rapidly than myristic acid. Myristic acid itself, and all longer-chain saturated acids, are recovered unchanged after oxidation and estimated by a second passage through the column. The amounts of unsaturated acids are then obtained by difference. This method is applicable to the analysis of the fatty acids in a wide variety of natural oils.

METHODS

Reversed-phase chromatography

A standard 1·3 × 30 cm. column of water-repellent kieselguhr, impregnated with liquid paraffin, was employed throughout (Howard & Martin, 1950). Each column was used repeatedly, but an ageing effect was apparent after some time; recoveries of pure acids then fell to under 95%. As soon as this occurred a fresh column was substituted.

Acetone–water mixtures containing 0·001% (w/v) bromothymol blue as indicator, and varying in acetone content from 35% (v/v) to 80% by 5% steps, were used for elution. The acetone (British Drug Houses Ltd.) was not specially purified and the solutions were always slightly acid, necessitating the subtraction of a small blank from the estimated acid equivalents.

Successive 2 ml. portions of eluate were titrated under N_2 with standardized 0·01 N-KOH in methanol contained in a 0·5 ml. Agla micrometer syringe which functioned as a burette. This enabled titrations to 0·001 ml. to be made with accuracy. Standard titrations on pure acids without passage through the column indicated that a blue-green end point (corresponding to pH 7·4 in aqueous solution) is necessary for complete neutralization in the higher acetone concentrations. Using this end point, pure stearic acid in 75% acetone gave an estimate of 95·4%, and hexanoic (caproic) acid in 35% acetone an estimate of 99·2%. A rate of flow of 2 ml. of eluate in 1·5–2 min. was found to give satisfactory separations.

No special precautions were taken to maintain a steady temperature, and this varied from 16 to 23° on different occasions. In any one experiment the temperature fluctuation was not greater than 2°.

Amounts of fatty acids from 1 to 60 mg. have been analysed with recoveries of over 95%.

Oxidation method

A sample of the fatty acid mixture, containing approximately 100 mg., was heated with 1 ml. 0·5 N-KOH in methanol and 5 ml. water. After evaporation of the methanol and cooling, 0·2 ml. of 50% (w/v) aqueous KOH and 15 ml. of 5% (w/v) $KMnO_4$ solution were added, and the mixture was set aside at room temperature for from 16 to 18 hr. At the end of this time 2 ml. of 50% (v/v) H_2SO_4 were

Table 1. *Source and data for fatty acids*

Fatty acid	M.p. (°)	I_2 value	Source	Literature reference
Oleic	12·3–13·1	89·1	Natural	Baker, Linstead & Weedon, unpublished
Elaidic	43·4–43·7	90·2	Isomerization of natural oleic acid	Bounds, Linstead & Weedon, 1953
Petroselinic	29·0–29·5	—	Synthetic	Baker, Kierstead, Linstead & Weedon, 1954
cis-Octadec-2-enoic	50·5–51·0	—	Synthetic	Myers, 1951
trans-Octadec-2-enoic	58	—	Synthetic	Myers, 1951
Erucic	—	—	—	Sample from Professor N. K. Adam
Linoleic[a]	—	178·2	—	Sample from Dr N. W. Hanson
α-Elaeostearic[b]	47·5	—	Natural	Crombie & Tayler, 1954
β-Elaeostearic[c]	72	—	Isomerization of natural α-elaeostearic	Crombie & Tayler, 1954
Stearolic	46·0–46·5	—	Synthetic	Baker, Linstead & Weedon, unpublished
Ximenynic	—	—	Natural	Ligthelm & Schwarz, 1950
Erythrogenic	—	—	Natural	Black & Weedon, 1953 Sample isolated by Dr E. M. Meade
erythro-9:10-Dihydroxystearic	131	—	Synthetic	Bounds *et al.* 1953
threo-9:10-Dihydroxystearic	92	—	Synthetic	Bounds *et al.* 1953
(+)-Ricinoleic	3	—	Partially synthetic	Crombie & Jacklin, 1954
α-Kamlolenic[d]	78	—	Natural	Crombie & Tayler, 1954
β-Kamlolenic[e]	89	—	Isomerization of natural α-kamlolenic	Crombie & Tayler, 1954
(+ −)-Tuberculostearic	24·1–24·5	—	Synthetic	Linstead, Lunt & Weedon, 1950

u.v. light absorption

[a] After alkali isomerization the specific extinction coefficient k^* (233 mμ.) = 91·5 (theor. 90·3).
[b] $\lambda_{max.}$ 261, 271, 281 mμ.: $\epsilon_{max.}$ 36 000, 47 000, 38 000.
[c] $\lambda_{max.}$ 259, 268, 279 mμ.: $\epsilon_{max.}$ 47 000, 61 000, 49 000.
[d] $\lambda_{max.}$ 261, 271, 282 mμ.: $\epsilon_{max.}$ 40 500, 52 000, 42 000.
[e] $\lambda_{max.}$ 258, 268, 279 mμ.: $\epsilon_{max.}$ 45 000, 60 000, 47 000.

* $k = \dfrac{\log_{10} I_0/I}{cl}$, where c = concentration in g./1000 ml., l = length of solution in cm. (Brice *et al.* 1952).

added, followed by concentrated $NaHSO_3$ solution until the permanganate was decolorized; the mixture was then warmed to just below boiling point. After cooling, the fatty acids were extracted with 3×15 ml. portions of light petroleum (b.p. 40–60°) and the combined extracts washed with 4×15 ml. portions of distilled water. The light petroleum was then evaporated from a suitable sample representing from 2 to 100 mg. of the original material. The residue was dissolved in 55% or 60% acetone and chromatographed on the column for estimation of the saturated acids present. Usually the original unsaturated acid was completely destroyed by oxidation, but in a few instances small amounts remained. The error caused by incomplete oxidation was estimated by determining the iodine value (Wijs) of the oxidized fatty acid. Less than 1% (calculated as oleic acid) of the original unsaturated acid was normally found; if a higher value was recorded the oxidation was repeated. Palmitic acid could be recovered from standard mixtures in 95–98% yield after oxidation, and myristic acid in 90–96% yield. Lauric acid gave somewhat lower recoveries and the method is therefore not applicable, in its present form, to saturated acids with less than 14 carbon atoms in the chain.

MATERIALS

Saturated straight-chain fatty acids. Highly purified specimens of lauric, myristic, palmitic, stearic and arachidic acids were obtained from Professor N. K. Adam, F.R.S.

Unsaturated and substituted acids. Data on the source and purity of the specimens used are given in Table 1. Each acid was checked for purity by the chromatographic method. Under 0·5% (calculated on total acid equivalent) of impurity can be detected in a 5 mg. sample providing that the R_F values are sufficiently different to ensure separation.

Neither linolenic nor licanic acid was isolated, but deductions were made from the chromatograms of the total fatty acids of linseed and oiticica oils respectively.

RESULTS

Saturated acids

Complete separation of straight-chain even-numbered saturated fatty acids was confirmed using the following eluting agents to bring off successive acid bands: C_8, 35% acetone; C_{10}, 40% or 45%; C_{12}, 50% or 55%; C_{14}, 55% or 60%; C_{16}, 65%; C_{18}, 70%; C_{20}, 75%. Recoveries (by titration) were above 95% for amounts of fatty acids in the range 1–60 mg.

Unsaturated acids

The majority of naturally occurring unsaturated acids are of the C_{18} series and only a single example (erucic acid) has been examined outside this. The groups investigated were mono-, di-, and triethylenic acids, acetylenic acids, and hydroxy- and keto-substituted acids. The results are summarized in Table 2.

Separation of mono-, di-, and tri-ethylenic acids

The results recorded in Table 2 show that all the C_{18} monoethylenic acids investigated can be separated from the C_{18} diethylenic acid, linoleic, and that in the absence of myristic acid a more or less complete separation of the C_{18} triethylenic acids from linoleic acid is possible. Particular importance attaches to the estimation of the widely distributed oleic and linoleic acids in the presence of saturated acids. Using mixtures containing different amounts of pure myristic, palmitic, oleic and linoleic acids, complete separation of myristic and linoleic acids from palmitic and oleic acids was obtained (Fig. 1; Table 3). The mixtures were then oxidized by the Bertram procedure and the products rechromatographed. After oxidation no acidic products are extracted from linoleic acid, while the oxidation products of oleic acid are eluted more rapidly than myristic acid. A complete separation of these oxidation products from the remaining saturated acids is therefore possible. After oxidation, palmitic and all longer-chain saturated acids give recoveries from 95 to 98% (by titration), myristic acid from 90 to 96%, and lauric acid from 88 to 92% (Crombie, Comber & Boatman, 1954). The method is therefore not accurate for the estimation of lauric acid. The latter can, however, be estimated before oxidation providing no other acid eluting at a similar rate is present. Linoleic acid can be estimated independently by ultraviolet light absorption following alkali isomerization (Brice, Swain, Herb, Nichols & Riemenschneider, 1952). The results obtained by this spectrophotometric method agree well with those from the oxidation method outlined above (see Table 4). An iodine value can also be used, in the absence of conjugated material, to estimate the total amount of unsaturation.

Both oleic and petroselinic acids are eluted with palmitic acid and both are broken down into shorter-chain fragments by the oxidation procedure. No quantitative estimate of oleic acid is possible when it is accompanied in a fatty acid mixture by petroselinic acid, but the presence of the latter acid is indicated immediately by its main oxidation product (lauric acid), which is eluted more slowly than the oxidation products of oleic acid.

Erucic acid is eluted at the same rate as arachidic acid but only the latter acid emerges in this position after oxidation. The oxidation products of erucic acid are eluted faster than myristic acid, and this unsaturated acid can therefore be determined in the presence of all saturated acids having a longer chain than myristic acid.

The separation of the C_{18} triethylenic acids from linoleic acid is possible only in the absence of significant amounts of myristic acid, but as large quantities of the latter acid are rare in natural oils, the method should be of use in a large number of cases. Ultraviolet light absorption methods are also available for the estimation of linolenic acid (after alkali isomerization) and for the elaeostearic acids. The results by chromatographic analysis for

Table 2. *Behaviour of pure fatty acids on standard reversed-phase columns*

Columns 1·3 × 30 cm. Approximately 5 mg. of acid used for chromatography. 100 mg. used for oxidation. Sep. = separable from, insep. = inseparable from. *c*, *t* in formulae indicate *cis* and *trans*.

Fatty acid	Formula	% acetone used for elution	Separation from other acids	% of original acid peak after oxidation	Oxidation products (Bertram method)
Monoethylenic acids					
Oleic acid	$CH_3.(CH_2)_7.\overset{c}{CH}{=}\overset{c}{CH}.(CH_2)_7.CO_2H$	65	Insep. palmitic acid	0·5	Eluted before myristic acid
Elaidic acid	$CH_3.(CH_2)_7.\overset{t}{CH}{=}\overset{t}{CH}.(CH_2)_7.CO_2H$	65	Insep. palmitic acid	0·0	Eluted before myristic acid
Petroselinic acid	$CH_3.(CH_2)_{10}.\overset{c}{CH}{=}\overset{c}{CH}.(CH_2)_4.CO_2H$	65	Insep. palmitic acid	0·0	Eluted before myristic acid
cis-Octadec-2-enoic acid	$CH_3.(CH_2)_{14}.\overset{c}{CH}{=}\overset{c}{CH}.CO_2H$	70	Insep. stearic acid, sep. palmitic and oleic acids	0·0	Palmitic acid
trans-Octadec-2-enoic acid	$CH_3.(CH_2)_{14}.\overset{t}{CH}{=}\overset{t}{CH}.CO_2H$	70	Insep. stearic acid, sep. palmitic and oleic acids	0·0	Palmitic acid
Erucic acid	$CH_3.(CH_2)_6.\overset{c}{CH}{=}\overset{c}{CH}.(CH_2)_{12}.CO_2H$	75	Insep. arachidic acid	0·6	Eluted before myristic acid
Diethylenic acid					
Linoleic acid	$CH_3.(CH_2)_4.CH{=}CH.CH_2.CH{=}CH.(CH_2)_7.CO_2H$	60	Insep. myristic acid	0·0	Eliminated before chromatography
Triethylenic acids					
Linolenic acid	$CH_3.CH_2.CH{=}CH.CH_2.CH{=}CH.CH_2.CH{=}CH.(CH_2)_7.CO_2H$	55 or 60	Sep. linoleic acid in 55% acetone	0·0	Eliminated before chromatography
α-Elaeostearic acid	$CH_3(CH_2)_3.CH{=}CH.CH{=}CH.CH{=}CH.(CH_2)_7.CO_2H$	60	Partially sep. linoleic acid	0·0	Eliminated before chromatography
β-Elaeostearic acid	$CH_3.(CH_2)_3.CH{=}CH.CH{=}CH.CH{=}CH.(CH_2)_7.CO_2H$	60	Partially sep. linoleic acid	0·0	Eliminated before chromatography
Acetylenic acids					
Stearolic acid	$CH_3.(CH_2)_7.C{\equiv}C.(CH_2)_7.CO_2H$	60	Insep. myristic acid	0·0	Eliminated before chromatography
Ximenynic acid	$CH_3(CH_2)_5.C{\equiv}C.CH{=}CH.(CH_2)_7.CO_2H$	55	Insep. stearolic acid (see text)	0·0	Eluted before myristic acid
Erythrogenic acid	$CH_2{=}CH.(CH_2)_4.C{\equiv}C.C{\equiv}C(CH_2)_7.CO_2H$	50	Sep. myristic acid	0·0	Eliminated before chromatography
Hydroxy acids					
threo-9:10-Dihydroxystearic acid	$CH_3.(CH_2)_7.CHOH.CHOH.(CH_2)_7.CO_2H$	60	Bad tailing. Insep. palmitic acid	—	—
erythro-9:10-Dihydroxystearic acid	$CH_3.(CH_2)_7\ CHOH.CHOH.(CH_2)_7.CO_2H$	60	Bad tailing. Insep. palmitic acid	0·0	Eluted before myristic acid
Ricinoleic acid	$CH_3\ (CH_2)_5.CHOH.CH_2.CH{=}CH.(CH_2)_7.CO_2H$	50	Sep. myristic acid	0·4	Eluted before myristic acid
α-Kamlolenic acid	$CH_2OH.(CH_2)_3.CH{=}CH.CH{=}CH.CH{=}CH.(CH_2)_7.CO_2H$	50	Sep. myristic acid	0·0	Eliminated before chromatography
β-Kamlolenic acid	$CH_2OH.(CH_2)_3.CH{=}CH.CH{=}CH.CH{=}CH.(CH_2)_7.CO_2H$	50	Sep. myristic acid	0·0	Eliminated before chromatography
Keto acid					
Licanic acid	$CH_3.(CH_2)_3.CH{=}CH.CH{=}CH.CH{=}CH.(CH_2)_4.CO.(CH_2)_2.CO_2H$	50	Sep. myristic acid	—	—
Branched-chain acid					
Tuberculostearic acid	$CH_3.(CH_2)_7.CH.CH_3.(CH_2)_8.CO_2H$	70	Insep. stearic and arachidic acids	—	—

a number of natural oils can be seen in Fig. 2 and Table 4.

Comparison is made in Table 4 of the results obtained by the present chromatographic method with results in the literature obtained by standard methods. In the case of linseed oil, comparative analyses are included from oils of similar iodine value. The oiticica oil used for analysis by the chromatographic method appears to have a low content of an α-licanic acid. This low figure is confirmed by ultraviolet spectrophotometry.

DISCUSSION

Certain general conclusions can be drawn from these experimental results with regard to the effect of structure on elution rate. A difference of two CH_2 units in a straight-chain even-numbered saturated fatty acid is sufficient for complete separation from its homologues. Branching of the chain as in tuberculostearic (10-methyloctadecanoic) acid, introduces another carbon atom into the molecule and in this case results in elution between the C_{18} and C_{20} saturated straight-chain acids with separation from neither.

The occurrence of a double bond in the fatty acid chain increases the elution rate to a greater or less extent according to its position. If the unsaturation occurs adjacent to the carboxyl group, little change is noticed (the octadec-2-enoic acids are eluted only slightly more rapidly than octadecanoic acid and are inseparable from the latter). If the unsaturation is further removed from the acidic group, as in oleic, petroselinic or erucic acids, the elution rate is more profoundly altered and corresponds approximately with that of the saturated acid having a chain length of two CH_2 less. Oleic (octadec-9-enoic) acid is eluted with palmitic (hexadecanoic) acid, erucic (docos-13-enoic) acid is eluted with

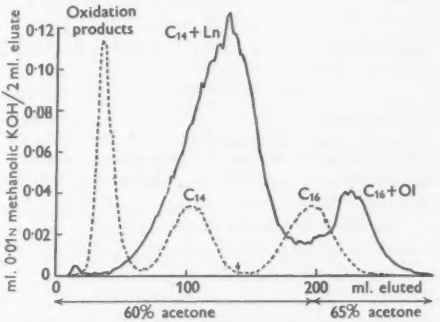

Fig. 1. Separation of linoleic (Ln) and myristic (C_{14}) acids from oleic (Ol) and palmitic (C_{16}) acids. Column 1·3 × 30 cm. Curves join titration points for every 2 ml. of eluate. ——, Chromatogram of mixture 2 (Table 3) before oxidation; - - -, chromatogram of mixture 2 after oxidation. Change from 60% acetone–water to 65% acetone–water indicated by arrow on the dotted curve. Amounts used are different for the two curves.

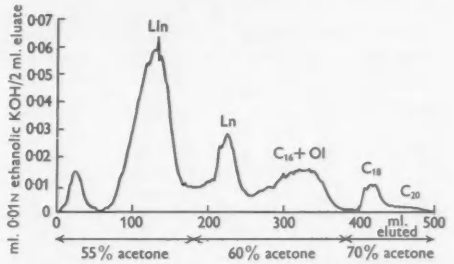

Fig. 2. Separation of the total fatty acids from linseed oil. Column 1·3 × 30 cm. Curve joins titration points for every 2 ml. of eluate. Lln = linolenic acid, C_{18} = stearic acid, C_{20} = arachidic acid. Other abbreviations as in Fig. 1.

Table 3. *Analysis of standard mixtures of pure acids by the chromatographic method*

	Linoleic	Myristic	Oleic	Palmitic	
Mixture 1					
% composition (wt.)	9·9	11·4	67·6	11·1	
% of total acid equivalent (calc.)	9·2	13·6	65·4	11·8	
		22·8		77·2	
% of total acid equivalent (found)		24		76	
% after oxidation (wt.)	—	10·0	—	10·8	
Mixture 2					
% composition (wt.)	71·2	9·2	10·4	9·2	
% of total acid equivalent (calc.)	69·0	10·9	10·3	9·8	(see Fig. 1)
		79·9		20·1	
% of total acid equivalent (found)		81·8		18·2	
% after oxidation (wt.)	—	8·2	—	8·9	
Mixture 3					
% composition (wt.)	25·8	23·7	26·6	23·9	
% of total acid equivalent (calc.)	24·0	27·2	24·5	24·3	
		51·2		48·8	
% of total acid equivalent (found)		47·7		52·3	
% after oxidation (wt.)	—	21·1	—	22·9	

Table 4. *Composition of fatty acids from natural oils*

The values are given as % by weight of total fatty acids

Fatty acid	Water melon: var. Tom Watson (U.S.A.) Chrom. method	Water melon: var. Cuban Queen (Florida, U.S.A.) Nolte & Loesecke (1939)	Palm kernel (W. African): Chrom. method	Palm kernel (W. African): Armstrong *et al.* (1925)	Palm kernel (W. African): Carsten *et al.* (1945)
Saturated					
C_6 or lower	—	—	1·0	—	—
C_8	—	—	4·3	3	4·3, 3·9
C_{10}	—	—	4·7	3	4·8, 6·3
C_{12}	—	—	46·0	52	51·3, 51·2
C_{14}	—	—	17·1	15	16·5, 17·5
C_{16}	10·1	9·1	9·9	7·5	7·6, 6·5
C_{18}	11·1	5·8	2·7	2·5	1·7, 2·0
C_{20}	0·6	0·8	Trace	—	0·6, 0·5
Unsaturated					
Oleic	7·2	13·5	13·3	16·0	11·3, 10·5
Linoleic	71·0 (68·9*)	70·8	1·0	1	1·3, 1·2
Linolenic	—	—	—	—	—
α-Licanic	—	—	—	—	—
I_2 value (Wijs)	138·0 (135·1†)	—	13·3 (13·8†)	—	—

Fatty acid	Linseed (samples with similar I_2 values): Chrom. method	Linseed: Gunstone & Hilditch (1946)	Linseed: Spectroscopy Committee Report (1948)	Oiticica: Chrom. method (sample from Dr N. H. E. Ahlers)	Oiticica: Kaufmann & Baltes (1936)	Oiticica: Machado (1938)	Oiticica: Martens (1952)
Saturated							
C_6 or lower		—	—	—	—	—	—
C_8	6·0‡	—	—	—	—	—	—
C_{10}		—	—	—	—	—	—
C_{12}		—	—	—	—	—	—
C_{14}	0·1	—	—	—	—	—	—
C_{16}	4·5	7		5·7		6·5	—
C_{18}	4·3	8	8·7	10·3	10·4	5·6	—
C_{20}	0·6	1		—		—	—
Unsaturated							
Oleic	15·1	13	24·1	14·3		4·5	—
Linoleic	15·3 (16·6*)	17	17·4	21·5	16·0	—	—
Linolenic	54·1 (53·6*)	54	49·4	—		—	—
α-Licanic	—	—	—	48·2 (48·4§)	73·6	80·8	50·05
I_2 value (Wijs)	197·0 (189·3†)	191·3	189·1 (Oil)	—	—	—	—

* By u.v. spectrophotometric method (Brice *et al.* 1952).
† Iodine value calculated from chromatographic results.
‡ Part of this acid peak is believed to be oxidation products of the unsaturated acids.
§ By u.v. spectrophotometry using extinction coefficient of Martens (1952). (Using value of Kaufmann *et al.* 1950, % licanic acid = 51·3.)

arachidic (eicosanoic) acid. The difference in position of the double bond in oleic (octadec-9-enoic) and petroselinic (octadec-6-enoic) acids causes no marked difference in elution rate. The *cis* and *trans* isomers of monoethylenic acids appear to be inseparable (e.g. oleic and elaidic acids; *cis*- and *trans*-octadec-2-enoic acids). Other geometrical isomers which have been tested are inseparable; for example α-elaeostearic acid (considered to be octadec-*cis*-9-*trans*-11-*trans*-13-trienoic acid; Bickford, DuPre, Mack & O'Connor, 1953; Paschke, Tolberg & Wheeler, 1953) is eluted at the same rate as the *all-trans* β isomer. The same is true of the kamlolenic acids, the α isomer of which is reported to be 18-hydroxyoctadec-*cis*-9-*trans*-11-*trans*-13-trienoic acid, and the β isomer the *all-trans* form (Crombie & Tayler, 1954).

The effect of a second double bond has been investigated in a single case only, that of linoleic acid. Here it results in an increase in elution rate, this acid moving slightly more slowly than myristic (tetradecanoic) acid although inseparable from it in all proportions. Complete separation from monoethylenic acids of the same chain length is possible.

The presence of a third double bond, in those acids examined, results in a further increase in elution

speed. Linolenic (octadec-9:12:15-trienoic) acid and the elaeostearic (octadec-9:11:13-trienoic) acids move faster than myristic acid.

An acetylenic link causes a very marked change in elution rate. Stearolic (octadec-9-ynoic) acid is eluted at the same rate as myristic acid and is completely separable from the corresponding monoethylenic acids (elaidic and oleic). One triple bond has therefore a greater effect than two double bonds. Successful separation of octadecanoic, octadec-9-enoic, and octadec-9-ynoic acids from each other is possible. The effect of an additional double bond in an acetylenic acid increases its elution rate only slightly (ximenynic acid is eluted rather faster than stearolic acid but is inseparable from it on a 1·3 × 30 cm. column). A second acetylene linkage as in erythrogenic (octadec-17-en-9:11-diynoic) acid causes very rapid elution.

The effect of a keto group has been investigated only in the case of licanic (4-oxo-octadec-9:11:13-trienoic) acid, and in this instance causes a marked increase in elution rate. Licanic acid is completely separable from the unsubstituted elaeostearic (octadec-9:11:13-trienoic) acids.

The presence of hydroxyl groupings in a fatty acid causes very speedy elution. Complete separation of ricinoleic (12-hydroxyoctadec-9-enoic) acid from oleic (octadec-9-enoic) acid, and of kamlolenic (18-hydroxyoctadec-9:11:13-trienoic) acid from elaeostearic (octadec-9:11:13-trienoic) acid has been achieved. The presence of two hydroxyl groups appears to reduce the solubility of the acids in paraffin so much that bad tailing results. The occurrence of significant quantities of such acids in a mixture would prevent successful chromatographic analysis.

Investigation of a large number of different unsaturated acids and of mixtures including saturated acids has shown that the method proposed (chromatograms before and after oxidation) is suitable for the analysis of a large number of fatty acids in milligram quantities. Chemical identification of acid fractions recovered from the columns has been carried out but, as very small quantities are involved, care is needed.

SUMMARY

1. The separation of representative unsaturated acids on a standard reversed-phase column has been investigated. A number of conclusions are drawn regarding the relationship between structure and elution rate.

2. A method is devised for the analysis of milligram quantities of fatty acid mixtures containing both saturated and unsaturated acids. The most widely distributed unsaturated fatty acids are separable from each other by the present chromatographic technique but not from certain saturated fatty acids which are eluted at similar rates. The latter are estimated by a second chromatogram following oxidation by alkaline permanganate, which removes the unsaturated material. The amounts of unsaturated fatty acids are then determined by difference.

3. The method has been tested on standard fatty acid mixtures, and on the fatty acids from a number of natural oils. It gives good agreement with other techniques.

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Studies Involving Enzymic Phosphorylation

3. THE PHOSPHORYLATION OF D-RIBOSE BY EXTRACTS OF *ESCHERICHIA COLI**

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Although several notable advances have recently been made in the elucidation of the intermediary metabolism of the pentose phosphates, little information is at present available regarding their enzymic formation from free pentoses. Sable (1950, 1952) showed that ribose disappeared when incubated with ammonium sulphate fractions prepared from extracts of baker's or brewer's yeast, supplemented with adenosine triphosphate (ATP), magnesium and fluoride ions. He detected the formation of hexose phosphates by specific enzymic methods, but obtained no evidence for the intermediate formation of a phosphate ester of ribose. Cohen & Raff (1951) found that when *Escherichia coli* was grown on ribose, the washed cells specifically utilized this pentose. Hochster & Watson (1952) reported that a cell-free extract of xylose-grown *Pseudomonas hydrophila*, when incubated with xylose, ATP, magnesium and fluoride ions, liberated carbon dioxide from a bicarbonate buffer under anaerobic conditions, an observation which was consistent with the postulated phosphorylation of the xylose by ATP, but the isolation of a xylose phosphate was not attempted. More recently, Lampen (1953) has shown that when a cell-free extract of xylose-grown *Lactobacillus pentosus* was incubated with xylose and ATP, an equilibrium mixture of ribose 5-phosphate and ribulose 5-phosphate was formed.

In the work to be described, a strain of *Esch. coli* has been grown under conditions similar to those of Cohen & Raff (1951). When the washed cells were disintegrated, the cell-free extract was found to catalyse the transfer of the terminal phosphate group of ATP to ribose when magnesium ions were present. The characteristics of this enzymic phosphorylation have been investigated.

* Part 2, Long (1953).

A preliminary account of this work has already appeared (Heald & Long, 1953).

EXPERIMENTAL

Materials

D-Ribose was obtained from Roche Products Ltd.; we have found it to be chromatographically pure. Other monosaccharides were the best available commercial preparations.

Adenosine triphosphate was isolated as the barium salt from rabbit muscle by the method of Dounce, Rothstein, Beyer, Meier & Freer (1948) and stored as a frozen 0·05 M solution of the potassium salt at $-20°$.

A sample of synthetic barium ribose 5-phosphate was generously given by Dr Gertrude Glock, Courtauld Institute of Biochemistry, Middlesex Hospital, London. Barium was removed by treating a solution in 0·01 N-HCl with a slight excess of solid K_2SO_4, centrifuging down the $BaSO_4$ and neutralizing the supernatant.

In all experiments *Esch. coli* (National Collection of Industrial Bacteria, no. 7271) was used. The cultures were maintained on slopes of percentage (w/v) composition: agar 2·67, yeast extract ('Oxoid') 0·1, bacterial peptone ('Oxoid') 0·1, $NH_4H_2PO_4$ 0·07, NaCl 0·17, glucose 0·13.

The buffer used throughout was aminotrishydroxymethyl methane (THAM), pH 7·6 (Gomori, 1946).

Methods

Growth of Esch. coli 7271. A loop inoculum from a slope was introduced into 5 ml. of liquid medium of percentage (w/v) composition: yeast extract 0·3, bacterial peptone 0·3, $NH_4H_2PO_4$ 0·2, NaCl 0·5, glucose 0·4, brought to pH 7·5 with NaOH. After 16 hr. growth at 38°, 0·2 ml. inoculum was transferred into each of about twenty-five flat bottles containing 15 ml. of solid culture medium of percentage (w/v) composition: agar 2, yeast extract 0·5, ribose 0·25, NaCl 0·07, K_2HPO_4 0·45, KH_2PO_4 0·12, $CaCl_2$ 0·0007, $MgCl_2$ 0·0007. In the preparation of this medium, the ribose was autoclaved separately and then added to the other sterilized components. After 16 hr. growth at 38°, the

bacteria were washed off the agar with a total of about 45 ml. 0·1 M THAM buffer, pH 7·6, and the suspension was filtered through gauze. The bacteria were centrifuged at 3° in an angle centrifuge at 8000 *g*, washed twice with buffer and finally suspended in buffer to a final concentration of 20 mg. dry wt./ml. Eight successive experiments gave an average yield of *Esch. coli* of 15·9 mg. dry wt./bottle (range 12·6–20·2); this is equivalent to slightly more than 1 g. dry wt. of bacteria/l. solid culture medium.

For comparative purposes, *Esch. coli* 7271 was also grown on solid medium containing glucose in place of ribose. The amount of growth was similar with these two sugars.

Preparation of cell-free extracts. About 2–3 g. of washed Pyrex ballotini (grade 12) were added to 5–10 ml. of buffered *Esch. coli* suspension, and the mixture shaken at 3° in a Mickle (1948) disintegration apparatus for 20 min. The suspension of broken cells was decanted from the ballotini, centrifuged at 3° and the cloudy supernatant frozen at −20°. When the latter was allowed to thaw, much inactive material flocculated and was removed by centrifuging. The final supernatant, which was still somewhat opalescent, was stored in the frozen state at −20°.

Utilization of ribose by Esch. coli 7271. In experiments with intact cells, 0·05–0·10 ml. *Esch. coli* suspension (20 mg. dry wt./ml.) was incubated at 38° for 10 min. in 0·08 M THAM buffer, pH 7·6, containing 1 μmole ribose in a total volume of 0·5 ml. The reaction mixture was then chilled to 0°, immediately diluted to 5·0 ml. with ice-cold water, mixed thoroughly and centrifuged at 3°. The ribose content of 1 ml. of clear supernatant was determined by the method of Mejbaum (1939), as modified by LePage (1949), using orcinol recrystallized from benzene. Initial ribose and blank determinations were made on unincubated cells.

When cell-free preparations were employed, 0·1 ml. of bacterial extract was incubated for 5–20 min. at 38° in a reaction mixture of total volume 0·5 ml. having the following composition (final concentrations in brackets): ribose (0·002–0·006 M), ATP (0·005 M), $MgCl_2$ (0·005 M), THAM buffer, pH 7·6 (0·04 M), with or without $(NH_4)_2SO_4$ (0·28 M). (The reason for including $(NH_4)_2SO_4$ is explained in the Results section.) Enzyme action was stopped by treating the incubated reaction mixture with 0·5 ml. 5% (w/v) $ZnSO_4$ followed by 0·5 ml. 0·3 N-$Ba(OH)_2$ (Somogyi, 1945), while 0·07 ml. 2 M barium acetate was also added to precipitate the excess sulphate ion, whenever $(NH_4)_2SO_4$ was used. In such cases the addition of the $ZnSO_4$ before the $Ba(OH)_2$ was necessary, for otherwise destruction of ribose by the liberated NH_4OH occurred. The mixture was diluted with water to 5 ml., centrifuged and the supernatant filtered through a no. 42 Whatman paper. This method of deproteinization is well known to precipitate adenine nucleotides and has also been found to adsorb ribose phosphates completely (see Table 1). Ribose concentration was determined on 1 ml. of the filtrate and on both a zero-time reaction mixture and a control without ribose, similarly treated.

When it was required to precipitate the ATP only and to determine the sum of the concentrations of ribose and pentose phosphate, enzyme action was stopped by addition of 0·3 ml. of 20% (w/v) mercuric acetate in 0·1% (v/v) acetic acid. The volume was made up to 5 ml. with 0·1% acetic acid, and after standing for about 30 min. to ensure complete precipitation, the mixture was centrifuged and the supernatant filtered; 1 ml. of the filtrate was taken for pentose determination. The presence of $(NH_4)_2SO_4$ in the reaction mixture was found to be advantageous, for it caused the simultaneous precipitation of a crystalline mercuric ammonium compound which assisted in carrying down the mercuric complexes of the adenine nucleotides. Separate experiments showed that ribose and ribose 5-phosphate were not removed from solution by the mercuric acetate treatment, while ATP was completely precipitated under these conditions. The presence of excess mercuric ions had no effect on the determination of the pentose content of the filtrate by the orcinol method. The concentration of pentose phosphate was taken as the difference between the total pentose concentrations of the mercury and barium–zinc filtrates. This mercury-precipitation procedure is based on the observations of Kerr (1940, 1941). The specificity of the mercury and barium–zinc precipitations is shown in Table 1.

Other analytical methods. Glucose and other hexoses were determined as reducing substances by the copper reduction–arsenomolybdate method of Nelson (1944). Phosphate was determined by the micromodification (Long, 1943) of the method of Berenblum & Chain (1938). Acid-labile phosphorus of ATP was determined after hydrolysis in N-$HClO_4$ for 10 min. on a boiling-water bath.

Table 1. *Determination of ribose, ribose 5-phosphate and adenosine triphosphate separately and in mixtures*

Solutions of ribose, ribose 5-phosphate (R 5-P) and ATP were used singly or in mixtures in total vol. 0·5 ml. In (*a*) the solution was diluted to 5·0 ml. with water; in (*b*) 0·3 ml. of 20% (w/v) mercuric acetate in 0·1% (v/v) acetic acid was added, followed by 0·1% acetic acid to 5·0 ml., and the mixture centrifuged and filtered; in (*c*) 0·5 ml. of 0·3 N-$Ba(OH)_2$ and 0·5 ml. of 5% (w/v) $ZnSO_4$ were added, followed by water to 5·0 ml., and the mixture was centrifuged and filtered. 0·4 ml. samples of filtrate were taken for pentose determination by the orcinol method. The calculated values are based on the assumption that treatment (*b*) precipitates ATP only, while treatment (*c*) precipitates both ATP and R 5-P.

Composition of original solution (μmoles)			Pentose (μmoles) found after					
			(*a*) No ppn.		(*b*) Mercury ppn.		(*c*) Barium–zinc ppn.	
Ribose	R 5-P	ATP	Obs.	Calc.	Obs.	Calc.	Obs.	Calc.
1·03	0	0	1·00	1·03	1·04	1·03	0·97	1·03
1·03	0·91	0	2·05	1·94	1·94	1·94	1·05	1·03
1·03	0	2·69	3·71	3·72	1·01	1·03	0·99	1·03
1·03	0·91	2·69	4·81	4·63	1·95	1·94	0·95	1·03
0	0·91	0	0·92	0·91	0·84	0·91	0·01	0
0	0·91	2·69	3·54	3·60	0·84	0·91	0	0
0	0	2·69	2·61	2·69	0	0	0	0

RESULTS

The utilization of monosaccharides by intact cells of Esch. coli 7271

Washed intact cells of glucose-grown and ribose-grown *Esch. coli* 7271 were found to utilize glucose, mannose and glucosamine rapidly, galactose less so and fructose only slowly. Ribose was fermented only by the cells grown on this pentose, while other pentoses were not utilized by either kind of cells.

In a Warburg experiment, using ribose-grown *Esch. coli* cells (4 mg. dry wt.) in 1 ml. 0·05 M THAM buffer, pH 7·6, and 2 μmoles of pentose, the O_2 uptakes (μl.) in 1 hr. were: no substrate, 43; D-xylose, 44; D-arabinose, 45; D-ribose, 166 and 167 (duplicate).

The utilization of ribose by cell-free preparations of Esch. coli 7271

Cells of ribose-grown *Esch. coli* 7271, suspended in 0·1 M THAM buffer, pH 7·6, were readily disintegrated in a Mickle (1948) apparatus. It was established by microscopic examination after Gram staining that this procedure gave complete disintegration of the cells. This preparation of broken cells was now able to utilize D-ribose only when supplemented with ATP and magnesium ions. When the glass ballotini and cell debris were removed by centrifuging at about 8000 ***g*** for 10 min. at 3°, the whole of the phosphorylating activity was found in the supernatant. Maximal rates of ribose utilization were obtained with 0·005 M-ATP. Similar active supernatants were obtained when the cells were disintegrated in the following media, pH 7·6; 0·12 M potassium phosphate buffer; 0·04 M veronal (King & Delory, 1940); 0·05 M bicarbonate containing 0·15 M-KCl. Other pentoses were not utilized under any of these conditions.

Fluoride, which was found necessary for inhibiting phosphatase activity in 'homogenates' of animal tissues in enzymic phosphorylation experiments (Case & McIlwain, 1951; Long, 1951), was not required. However, the extracts had slight adenosinetriphosphatase activity. When present at a concentration of 0·05 M, potassium fluoride was found to inhibit ribose disappearance by about 25 %. Sodium iodoacetate (0·01 M) also inhibited to about the same extent. Some of these results are shown in Table 2.

Table 2. *Utilization of* D-*ribose by extracts of ribose-grown* Esch. coli

The complete system contained 0·1 ml. of extract of ribose-grown *Esch. coli* (derived from 2 mg. dry wt. of cells), 0·005 M ribose, 0·005 M-ATP, 0·005 M-$MgCl_2$ and 0·02 M THAM buffer, pH 7·6; total vol. 0·5 ml. 30 min. at 38°.

	Ribose disappearing (μmoles)
Complete system	1·38
Same without $MgCl_2$	0·06
Same without ATP	0·00
Same with 0·01 M iodoacetate	1·11
Same with 0·05 M-KF	1·04

Dialysis in a collodion sac for 2·5 hr. at 4° against 0·1 M THAM buffer, pH 7·6, in one experiment caused a 10 % loss of activity, which was not restored by addition of an equal volume of the boiled extract from an undialysed preparation. In another experiment, carried out under similar conditions, no loss of activity was observed. Thus there is no evidence for the requirement of any dialysable coenzyme.

The enzymic formation of a pentose phosphate from ribose by extracts of Esch. coli

Duplicate reaction mixtures containing ATP, ribose and magnesium ions were incubated with an extract of ribose-grown *Esch. coli*, under conditions similar to those described in Table 2. One sample was treated with the $Ba(OH)_2$–$ZnSO_4$ reagents of Somogyi (1945) and the filtrate analysed for pentose; the other was treated with mercuric acetate in acetic acid and the sum of the free and phosphorylated pentose was determined in the filtrate. The fraction of the metabolized ribose which could be accounted for in terms of phosphorylated pentose was thus determined.

When ribose-grown *Esch. coli* cells were freshly disintegrated and the suspension centrifuged, the fraction accumulating as pentose phosphate in the presence of the supernatant extract was usually only about 0·5–0·6. However, when the extract was frozen and thawed several times and insoluble material removed by centrifuging, the fraction accumulating as pentose phosphate was appreciably increased, although the rate of utilization of free pentose was practically unchanged. It seems, therefore, that the enzyme systems leading to the breakdown of pentose phosphates are either destroyed by storage or else become insoluble on freezing and thawing and are then removed by centrifuging.

It has been found that in the presence of high concentrations of ammonium sulphate the fraction of the ribose disappearing which accumulates as pentose phosphate is uniformly high and does not differ from unity within the experimental error of the determinations. This is shown in Table 3, from which it is seen that the best results are obtained with 0·28 M ammonium sulphate. The ammonium sulphate appears to act by inhibiting the breakdown of the accumulated pentose phosphate, a view which is supported by the finding that the rate of disappearance of a sample of added ribose 5-phosphate was markedly decreased in the presence

Table 3. *Accumulation of pentose phosphate after enzymic phosphorylation of ribose by ATP in the presence of extracts of ribose-grown* Esch. coli

The reaction mixtures contained 0·1 ml. of extract of ribose-grown *Esch. coli* (derived from 2 mg. dry wt. of cells), 0·005 M-ATP, 0·005 M-$MgCl_2$ and 0·04 M THAM buffer, pH 7·6, with or without added salts at the concentrations specified. Ribose concentration 0·004 M (Expt. 1), 0·002 M (Expt. 2); total vol. 0·5 ml. Incubated at 38° for 20 min. (Expt. 1) or 14 min. (Expt. 2). Ribose was determined in $Ba(OH)_2$–$ZnSO_4$ filtrates; the sum of the ribose and pentose phosphate was determined in mercuric acetate filtrates.

Expt. no.	Salt added	Concn. (M)	Ribose utilized (μmoles)	Pentose phosphate accumulated (μmoles)	Fraction accumulating as pentose phosphate
1	None	—	1·60	1·35	0·84
	$(NH_4)_2SO_4$	0·10	1·68	1·44	0·86
	$(NH_4)_2SO_4$	0·20	1·42	1·33	0·94
	$(NH_4)_2SO_4$	0·28	1·40	1·44	1·04
	$(NH_4)_2SO_4$	0·40	1·20	1·25	1·04
2	None	—	0·91	0·42	0·46
	$(NH_4)_2SO_4$	0·28	0·79	0·73	0·92
	K_2SO_4	0·28	0·98	0·83	0·83
	Na_2SO_4	0·28	0·56	0·48	0·86

Table 4. *Dependence of extent of ribose utilization and pentose phosphate accumulation on the amount of adenosine triphosphate present*

The reaction mixtures contained 0·1 ml. extract of ribose-grown *Esch. coli* (derived from 2 mg. dry wt. of cells) in 0·04 M THAM buffer, pH 7·6, 0·005 M-$MgCl_2$, 0·28 M-$(NH_4)_2SO_4$, 0·002 M ribose and the amounts of ATP shown. The reactions were allowed to proceed to completion (30 min.). Control experiments in which incubation was continued for 60 min. showed no increased disappearance of ribose. Ribose was determined in $ZnSO_4$–$Ba(OH)_2$ filtrates; ribose plus pentose phosphate was determined in mercuric acetate filtrates. The amounts of ATP were determined on separate samples by measurement of acid-labile phosphorus and assuming two atoms of acid-labile P/molecule of ATP.

Expt. no.	Adenosine triphosphate (μmole)	Ribose utilization (μmole)	Pentose phosphate accumulation (μmole)
1	0·74	0·65 (0·64*)	0·74 (0·61*)
	0·49	0·47	0·46
	0·25	0·13	0·15
2	0·31	0·32 (0·29*)	0·37 (0·32*)
	0·15	0·11	0·13
	0·08	0·02	0·03

* Values found in presence of a mixture of 0·008 M-KF and 0·00012 M adenosine monophosphate, which strongly inhibits myokinase activity.

of 0·28 M ammonium sulphate. Thus, high yields of accumulated pentose phosphate may be obtained in the presence of ammonium sulphate with either fresh or aged extracts of *Esch. coli*.

It is probable that the sulphate ion is responsible for this inhibition of the breakdown of pentose phosphate, for, as also shown in Table 3, both sodium and potassium sulphates produced qualitatively similar effects. Unfortunately, the effects of other anions could not be properly assessed, for when ammonium sulphate was replaced by ammonium chloride or ammonium acetate, the method of determining free plus phosphorylated pentose in the mercuric acetate filtrates failed to work.

Enzymic phosphate transfer from adenosine triphosphate to ribose

The need for the presence of ATP and magnesium ions in reaction mixtures for ribose utilization by extracts of ribose-grown *Esch. coli* (Table 2) may be taken as evidence that the reaction is a typical enzymic phosphorylation. Further support for this is provided by the fact that the extent of ribose disappearance and pentose phosphate accumulation are dependent on the amount of ATP present, as shown in Table 4. It is clear from these experiments also that one molecule of ATP is required for each molecule of ribose utilized, in contrast with the results of Hochster & Watson (1952) with *Pseud. hydrophila* and Lampen (1953) with *Lb. pentosus*, where the presence of myokinase in the bacterial extracts was found to bring about the overall transfer of both energy-rich phosphate groups of ATP in the phosphorylation of xylose. Furthermore, the amount of ribose disappearing and pentose phosphate accumulating is unaffected by the addition of 0·008 M fluoride and 0·00012 M adenosine monophosphate; this combination is known to inhibit strongly the myokinase reaction in animal tissues (Slater, 1953).

The equivalence of the phosphate transferred from ATP to ribose is shown by the experiment of Table 5. In the absence of ribose, a small amount of acid-labile phosphorus was found to disappear during the incubation while an approximately equivalent amount of inorganic phosphate was formed. This would be expected if the bacterial extract contained adenosinetriphosphatase, for the conditions of the experiment were likely to favour the activity of this enzyme, i.e. presence of

magnesium ions and absence of fluoride. Some acid-stable phosphorus was also formed during the incubation, but the source of this material has not been determined. These values were used for correcting the data obtained when ribose was present in the reaction mixtures. Under the latter conditions, both the loss of acid-labile phosphorus and gain of acid-stable phosphorus were much increased, and when corrected for the control values, there was equivalence within experimental error. The amount of ribose disappearing slightly exceeded the corrected amounts of phosphate transferred, but this would be expected since ammonium sulphate was not present in these reaction mixtures so that some decomposition of pentose phosphate would inevitably take place.

Table 5. *Phosphate equivalence between adenosine triphosphate utilized and pentose phosphate accumulated*

Ribose (2·0 μmoles) and ATP (2·5 μmoles) were incubated with 0·1 ml. extract of ribose-grown *Esch. coli* (derived from 2 mg. dry wt. of cells) in 0·04 M THAM buffer, pH 7·6, and 0·005 M-$MgCl_2$ for 20 min. at 38°; total vol. 0·5 ml. One reaction mixture was treated with the $ZnSO_4$–$Ba(OH)_2$ reagents of Somogyi (1945) and free ribose determined in the filtrate; another reaction mixture was deproteinized with $HClO_4$ (final concn. 5%) and the filtrate analysed for inorganic, acid-labile (10 min. at 100° in N-$HClO_4$) and total phosphorus. A control reaction mixture without ribose was incubated under similar conditions and the $HClO_4$ filtrate analysed for phosphate fractions. Zero-time reaction mixtures were analysed for initial ribose and phosphate fractions.

	Change in amount after 20 min. incubation		Difference due to ribose (μmole)
	Ribose absent (μmole)	Ribose present (μmoles)	
Inorganic P	+0·54	+0·66	+0·12
Acid-labile P	−0·55	−1·50	−0·95
Acid-stable P	+0·42	+1·30	+0·88
Ribose	—	−0·98	−0·98

The experiment in Table 5 is interpreted as demonstrating the conversion of acid-labile phosphorus of ATP into acid-stable pentose phosphate. It does not distinguish between (*a*) the transfer of the terminal group of ATP to ribose to give an acid-stable pentose monophosphate and (*b*) the transfer of both acid-labile groups of ATP or the terminal groups of two molecules of ATP to ribose to give a pentose diphosphate containing one acid-labile and one acid-stable phosphate group. This is due to the fact that the phosphate determinations were carried out on perchloric acid filtrates which contained both the pentose phosphate and the nucleotides. In view of the finding by Klenow (1953) of ribose 1:5-diphosphate as a coenzyme-intermediate of the phosphoribomutase reaction, it became necessary to distinguish between these possibilities.

In another experiment, therefore, phosphate determinations were made on mercury filtrates, which contain the pentose phosphate uncontaminated by nucleotides. It was found that the formation of 0·74 μmole of pentose phosphate, determined as the difference between the pentose-reacting substances in mercury and $ZnSO_4$–$Ba(OH)_2$ filtrates, was accompanied by the production of 0·73 μmole of acid-stable organic phosphate. No acid-labile phosphate was present. Thus it can be concluded that a pentose monophosphate is the product of the enzymic phosphorylation of ribose by ATP.

Absence of heptulose derivatives from reaction mixtures containing the products of phosphorylation of ribose by ATP in extracts of ribose-grown Esch. coli

The conversion of ribose 5-phosphate by enzymes of liver and spinach (Horecker, Smyrniotis & Klenow, 1953) and of erythrocytes (Dische & Pollaczek, 1952) into sedoheptulose 7-phosphate raised the possibility that phosphorylated seven-carbon sugars might be formed secondarily in the present experiments, following the initial enzymic

Table 6. *Optical densities at 580 and 670 mμ. of orcinol-reaction products of pentoses and of the product of enzymic phosphorylation of ribose by* Esch. coli *extracts*

1·0 μmole of ribose was incubated for 20 min. at 38° with 0·1 ml. extract of ribose-grown *Esch. coli* (derived from 2 mg. dry wt. of cells) in 0·04 M THAM buffer, pH 7·6, 0·005 M-ATP, 0·005 M-$MgCl_2$ and 0·28 M-$(NH_4)_2SO_4$. Pentose-reacting material was absent from $Ba(OH)_2$–$ZnSO_4$ filtrates. 1·0 ml. of the mercuric acetate filtrate was treated with the orcinol reagent and found to contain 0·192 μmole of pentose phosphate corresponding to a total yield of 0·96 μmole. This solution was examined in a Unicam SP. 500 spectrophotometer at 580 and 670 mμ. against an unincubated control free from ribose and an unincubated reaction mixture containing ribose, both subjected to similar mercuric acetate precipitations, followed by the orcinol reaction.

	Amount (μmole)	(*a*) $E_{580\ m\mu}$	(*b*) $E_{670\ m\mu}$	Ratio *b*/*a*
Ribose	0·200	0·123	0·463	3·76
Enzymically produced pentose phosphate	0·192	0·117	0·441	3·76
Arabinose	—	—	—	3·78*
Sedoheptulosan	—	—	—	0·29*

* Data of Horecker *et al.* (1953).

phosphorylation of ribose. The fact that sedoheptulose and its derivatives react in the orcinol test enabled us to investigate this possibility. Horecker *et al.* (1953) have reported that after carrying out the orcinol reaction, the ratio $E_{670\ m\mu}$: $E_{580\ m\mu}$ for pentoses is 3·78, while for sedoheptulosan it is 0·29. In the experiment reported in Table 6, ribose was incubated with ATP and *Esch. coli* extract under the usual conditions until all the free ribose had disappeared, as shown by the absence of pentose-reacting material in the $ZnSO_4$–$Ba(OH)_2$ filtrates. A sample of the mercury filtrate, containing pentose phosphate, was then subjected to the orcinol reaction and the optical densities at 580 and 670 mμ. were determined. As will be seen from Table 6, the observed ratio was in good agreement with the value reported by Horecker *et al.* (1953) for arabinose and with that found by us for ribose. Thus, it can be concluded that no seven-carbon sugar is formed under the conditions of our experiments.

DISCUSSION

When *Esch. coli* 7271 was grown on a medium with D-ribose as the carbon source, the washed cells were able to utilize ribose but not other pentoses. Disintegration of a suspension of the cells in buffer yielded an extract which also had the power to utilize ribose, always provided that adenosine triphosphate and magnesium ions were present in the reaction mixture. We have shown by the use of a precipitant, consisting of mercuric acetate in dilute acetic acid, that it is possible to remove the adenine nucleotides from an incubated reaction mixture and at the same time to leave in solution both unchanged ribose and its reaction products. The pentose content of such a mercuric acetate filtrate was always greater than the amount of free ribose, as determined in a filtrate from a similar reaction mixture after barium hydroxide–zinc sulphate precipitation, the difference between the two quantitatively representing an accumulation of pentose-containing products. It follows that under the conditions employed, the rate of formation of pentose-containing products exceeds their rate of degradation. This appears not to have been the case in the experiments of Sable (1950, 1952), in which no pentose-containing products could be detected following the action on ribose of a protein fraction prepared from an extract of yeast, supplemented with adenosine triphosphate and magnesium ions. The rate of removal of the pentose-containing products is low in our experiments, perhaps partly through the deliberate ageing of the extracts and because the enzymes involved become insoluble after freezing and thawing. However, the major factor responsible for the depression of their rate of removal is undoubtedly the presence of the high concentration of ammonium sulphate which was added to the reaction mixture, and which seems to inhibit the activity of the enzymes involved. Lampen (1953) also achieved some success in inhibiting ribose 5-phosphate degradation by cell-free extracts of *Lb. pentosus*, both by working at pH 8·0 and by a preliminary treatment of the enzyme extract with manganous chloride, followed by removal of the precipitate.

It appears that the reaction being studied is a simple enzymic transfer of the terminal phosphate group from adenosine triphosphate to ribose, giving a pentose monophosphate as the reaction product. This follows from the demonstration that there is equivalence between the amounts of ribose and acid-labile phosphate disappearing and of acid-stable phosphate accumulating. This work incidentally proves also that the pentose phosphate which is formed is not an aldopentose 1-phosphate, for such compounds are highly acid-labile. In the next paper of this series (Long, 1955), it will be shown that the product is in fact ribose 5-phosphate.

SUMMARY

1. Washed cells of ribose-grown *Esch. coli* 7271 are able to utilize ribose *in vitro*.

2. Cell-free extracts prepared from these bacteria will also bring about the disappearance of ribose when supplemented with adenosine triphosphate and magnesium ions.

3. The reaction consists of the enzymic transfer of the terminal phosphate group of adenosine triphosphate to ribose, yielding an acid-stable pentose monophosphate.

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Studies Involving Enzymic Phosphorylation

4. THE CONVERSION OF D-RIBOSE INTO D-RIBOSE 5-PHOSPHATE BY EXTRACTS OF *ESCHERICHIA COLI*

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In the previous paper of this series (Heald & Long, 1955), the enzymic phosphorylation of D-ribose by extracts of ribose-grown *Escherichia coli* in the presence of adenosine triphosphate and magnesium ions was described. Large-scale experiments under optimal conditions have now been carried out and the product of phosphorylation has been isolated as the barium salt. Although the substance obtained is not completely pure, its analysis, properties and reactions identify it as the barium salt of ribose 5-phosphate. A preliminary report of some of this work has already been given (Heald & Long, 1953).

EXPERIMENTAL

Materials

In addition to the D-ribose, adenosine triphosphate (ATP), synthetic barium ribose 5-phosphate and *Esch. coli* strain 7271, already described (Heald & Long, 1955), the following ribose phosphates were also studied for comparison:

Ribose 5-phosphate. This sample was obtained by hydrolysing dibarium ATP (1·7 g.) in 0·5N-H_2SO_4 (25 ml.) for 4·5 hr. on a boiling-water bath; excess barium acetate was added and $BaSO_4$ removed. Barium phosphate was precipitated by raising the pH to 8·0 with 10N-NaOH and barium ribose 5-phosphate was precipitated from the filtrate by addition of 4 vol. ethanol; purification was effected by solution in 0·01N-HCl and reprecipitation with 4 vol. of ethanol.

Ribose 2- and 3-phosphates. A mixture of the 2- and 3-phosphates of ribose (previously considered to be ribose 3-phosphate only) was prepared by acid hydrolysis of adenosine 3′-phosphoric acid (L. Light and Co. Ltd.) by the procedure of LePage & Umbreit (1943). The material was isolated as the barium salt.

Methods

The procedures for growing, washing and distintegrating the bacteria have already been described (Heald & Long, 1955); pentose was determined by the method of Mejbaum (1939) and phosphorus by the method of Long (1943).

The action of alkaline phosphatase on pentose phosphates. Alkaline phosphatase was prepared from rabbit intestinal mucosa by the *n*-butanol method of Morton (1950). The enzyme solution was dialysed against 0·025M ammonium acetate buffer, pH 8·9, and stored in the frozen state at −20°. When incubated at 37° for 15 min., 0·1 ml. of a 1 in 50 dilution of this stock enzyme solution liberated 0·15 μmole of inorganic phosphate from a reaction mixture containing 0·0125M diethylbarbiturate (veronal) buffer, pH 9·2 (King & Delory, 1940), 0·01M-$MgCl_2$ and 0·015M disodium β-glycerophosphate.

The enzymic hydrolysis of the pentose phosphates was studied as follows: a weighed amount of the barium salt (about 8 μmoles) was dissolved in 0·4 ml. 0·01N-HCl and the barium precipitated by addition of about 5 mg. of solid K_2SO_4. The suspension was centrifuged and the residue washed with 0·1 ml. 0·01N-HCl. The combined supernatants were adjusted to pH 9·2 with N-KOH and to the solution were added 0·016 ml. 0·5M-$MgCl_2$, 0·1 ml. 0·025M veronal buffer, pH 9·2, and 0·2 ml. of the stock phosphatase solution. The reaction mixture was incubated at 37° for 45 min., during which time a white precipitate, which was possibly $MgNH_4PO_4$, separated out. The tube was placed in a boiling-water bath for 5 min. to inactivate the phosphatase and then centrifuged. The clear supernatant was used for pentose analysis and for enzymic phosphorylation experiments.

Reducing power of ribose phosphates. Samples of the barium salts of the ribose phosphates (about 0·2 μmole; amount determined accurately as total pentose) were dissolved in 0·05–0·10 ml. 0·01N-HCl and the reducing power determined by the method of Nelson (1944). The colours were read in a photoelectric colorimeter (Evans Electroselenium Ltd.) against a ribose standard and water blank treated similarly.

Acid-lability of ribose phosphates. When ribose phosphates are heated on a boiling-water bath in N-$HClO_4$, both inorganic phosphate and free ribose are liberated. Ribose 5-phosphate, however, is much less acid-labile than is a mixture of ribose 2- and 3-phosphates. The procedure was as follows: to a known amount of the barium salt of the

appropriate pentose phosphate (about 1 μmole), dissolved in 0·2 ml. 0·01 N-HCl contained in a graduated centrifuge tube, were added 1·45 ml. water and 0·15 ml. 72% $HClO_4$ (giving a final concn. of N-$HClO_4$). The solution was heated on a boiling-water bath for a predetermined period not exceeding 7 hr. After the requisite time, the tube was cooled, and the contents neutralized to pH 7 (internal indicator, bromothymol blue) with 40% (w/v) NaOH and diluted to 2·5 ml.; a 0·5 ml. sample was taken for inorganic phosphate determination. The remaining 2 ml. were treated with 0·5 ml. 5% (w/v) $ZnSO_4$ and 0·5 ml. 0·3 N-$Ba(OH)_2$ (Somogyi, 1945), diluted with water to 5 ml., mixed, centrifuged and filtered; 3·0 ml. of clear filtrate were taken for free-ribose determination. Total ribose and total phosphorus were determined on samples of the unhydrolysed ribose phosphates. Zero-time samples, standards and blanks were analysed simultaneously.

Bromine oxidation. This method has been used to detect and determine ketose derivatives in the pentose phosphate samples, and is based on the procedure of Lampen (1953). However, the $BaCO_3$ used by the latter author was replaced here by $MgCO_3$. The final procedure is described in Table 3.

Periodate oxidation. The experimental procedure is described in Table 6.

Spectrophotometric determinations. Contamination by purine derivatives was tested for by examining the ultraviolet absorption of the pentose phosphates over the range 230–290 mμ. (Kalckar, 1947).

The presence of ketopentoses and of heptoses was tested for by carrying out the orcinol reaction of Mejbaum (1939) and examining the green solution over the range 500–700 mμ. In addition to the usual peak given by aldopentoses at 670 mμ., ketopentoses show a subsidiary peak at 540 mμ. (Horecker, Smyrniotis & Seegmiller, 1951); ketoheptoses, when treated in this way, do not possess an absorption band at 670 mμ. but instead show maximum absorption at 580 mμ. (Horecker, Smyrniotis & Klenow, 1953).

A Unicam spectrophotometer SP. 500 was used for these studies, using 1 cm. cells.

RESULTS

Large-scale enzymic phosphorylation of D-*ribose*

About ten large-scale enzymic phosphorylations have been carried out, with varying yields and purities of the product. In the earliest experiments, ammonium sulphate was not present in the enzyme reaction mixtures, with the result that the yields of pentose phosphate were low (Heald & Long, 1955), and the material isolated was grossly contaminated with phosphorus-containing products of further metabolism. When ammonium sulphate was later included in the reaction mixtures, however, the presence of the sulphate ion introduced an additional problem, namely, the separation of barium pentose phosphate from barium sulphate in the barium-fractionation procedure. It was eventually found that the best method of purification consisted in removing the adenine nucleotides by mercuric acetate precipitation and then adding barium acetate at a slightly alkaline pH, whereby the barium pentose phosphate became strongly adsorbed upon the barium sulphate precipitate. The pentose phosphate was then eluted by several treatments with dilute acid and finally purified by barium and ethanol fractionation. The most successful procedure is described below.

A cell-free extract of ribose-grown *Esch. coli* was prepared by the method given in the previous paper (Heald & Long, 1955); the extract was 0·1 M with respect to aminotrishydroxymethylmethane (THAM) buffer, pH 7·6, and 1 ml. contained the soluble material derived from 20 mg. dry wt. of bacteria. Before use, it was dialysed for 70 min. at 3° in a collodion sac against 8 vol. of 0·1 M THAM buffer, changing the buffer after 35 min. and stirring occasionally.

The dialysed enzyme (30 ml.) was incubated for 45 min. at 38° in a total volume of 150 ml. with a reaction mixture of the following composition (final concentrations in brackets): THAM buffer, pH 7·6 (0·04 M); $MgCl_2$ (0·005 M); potassium ATP (0·005 M); $(NH_4)_2SO_4$ (0·28 M) and D-ribose (0·005 M). Mercuric acetate (20% (w/v) in 0·1% (v/v) acetic acid; 90 ml.) was then added and the mixture kept overnight at 2°. After centrifuging, the residue was washed twice with 100 ml. portions of ice-cold 0·5% (w/v) mercuric acetate in 0·1% (v/v) acetic acid. The original supernatant and washings were combined and filtered; H_2S was passed through the clear filtrate for 30 min. The HgS precipitate was spun down and discarded, and the supernatant was filtered. The clear filtrate was evaporated *in vacuo* to about 50 ml. A sample of this solution on analysis, indicated that the total volume contained 232 μ-moles ribose and 450 μmoles pentose phosphate.

Isolation and purification of the product of enzymic phosphorylation

The solution, together with 5 ml. washings, was transferred to a 250 ml. centrifuge bottle. Barium acetate (2 M; 15 ml.) in slight excess was added, followed by 2·5 N-NaOH (about 12 ml.) until just alkaline to phenolphthalein. The mixture was centrifuged and the residue washed 3 times with 20 ml. portions of 0·1 M barium acetate made pink to phenolphthalein. The supernatant and washings on analysis were found to contain only free pentose and were therefore discarded.

The barium sulphate precipitate was extracted 3 times with 20 ml. portions of 0·1 N-HCl, the suspensions being centrifuged in an angle centrifuge at 8000 *g* for 15 min. each time. The supernatants were then filtered. Most of the pentose phosphate was present in the second fraction; a total of 287 μmoles pentose phosphate were recovered in this way.

The combined HCl supernatants (55 ml.) were treated with 1 ml. of 2 M barium acetate and 40%

(w/v) NaOH (about 1 ml.) until pink to phenolphthalein. The mixture was chilled overnight and the insoluble residue, which contained little pentose-reacting material, was discarded. The 'barium soluble' fraction was acidified to pH 6·0 and treated with 4 vol. ethanol. After chilling for 2 hr., the 'barium soluble–alcohol insoluble' material was spun down; it contained 197 μmoles pentose phosphate. The 'barium soluble–alcohol soluble' supernatant contained practically no pentose-reacting material and was discarded.

The 'barium soluble–alcohol insoluble' precipitate was dried *in vacuo* at room temperature, to remove ethanol, and dissolved in 10 ml. 0·1 N-HCl. Barium acetate (2 M; 0·1 ml.) was added and N-NaOH until the solution reacted just pink to phenolphthalein. The mixture was chilled for 1·5 hr., the small amount of precipitate spun down and discarded, the supernatant adjusted to pH 6·0 with conc. HCl and 40 ml. ethanol were added. After 2 hr. at 3°, the precipitate was spun down and washed with 10 ml. ethanol followed by 10 ml. ether. It was dried *in vacuo* at room temperature. Yield, 115·1 mg. of barium salt. On analysis, 1·485 mg. contained 2·29 μmoles pentose-reacting material, 2·75 μmoles organic phosphorus and 0·13 μmole inorganic phosphorus. When a sample was subjected to acid hydrolysis (N-$HClO_4$ on a boiling-water bath for 10 min.), it was found to contain about 10 % of its organic phosphorus in an acid-labile form; a sample of synthetic ribose 5-phosphate, by contrast, contained no detectable acid-labile phosphorus under these conditions. The contaminating organic phosphate was believed to have originated from the enzyme extract used.

The crude biosynthetic barium pentose phosphate (110 mg.) was dissolved in a mixture of 0·9 ml. 72 % $HClO_4$ and 9·9 ml. water, and the solution heated on a boiling-water bath for 1 hr. After cooling and neutralizing with 10 N-NaOH, 0·2 ml. 2 M barium acetate was added, the pH adjusted to 8·3 (pink to phenolphthalein) and the mixture placed in a refrigerator overnight. After centrifuging down the precipitate, which consisted largely of barium phosphate and was discarded, the supernatant was treated with 3 vol. ethanol and chilled. The 'barium soluble–alcohol insoluble' material was washed with 30 ml. ethanol, followed by 30 ml. ether. The product was dried *in vacuo* at room temperature. The yield was very low (about 35 mg.), but this was partly due to a mechanical loss.

On analysis, 1·60 mg. contained 2·94 μmoles of bound ribose and 3·41 μmoles total phosphorus. There was no inorganic phosphate and no acid-labile (10 min. in N acid at 100°) phosphorus. However, the pentose:phosphate ratio of 0·86 indicates that the expected purification had not been achieved and that there was present some acid-stable phosphate contaminant. The material gave no absorption in the 260 mμ. region, and was taken to be free from any contamination with purine derivatives. No further efforts were made to purify the material owing to the small amount available.

Properties and reactions of the biosynthetic barium pentose phosphate

The biosynthetic pentose phosphate, authentic ribose 5-phosphate and the mixture of ribose 2- and 3-phosphates when examined by paper chromatography, using different solvent systems invariably gave identical R_F values. As a result, this method could not shed any light on the position of the phosphate group in the biosynthetic material.

Examination for possible contamination by sedoheptulose 7-phosphate. The product of enzymic phosphorylation has been examined spectrophotometrically for contamination by seven-carbon sugar derivatives, although it has been shown in the previous paper (Heald & Long, 1955) that in small-scale experiments such contamination was not detectable.

The results of an experiment to test for sedoheptulose contamination (Table 1) do not provide any spectrophotometric evidence to suggest that this impurity is present in the biosynthetic pentose phosphate. For comparison, the results obtained with the sample of synthetic barium ribose 5-phosphate are also given.

The nature of the pentose in the biosynthetic pentose phosphate. So far, no evidence has been presented regarding the nature of the pentose in the biosynthetic product. The pentose itself was found to be readily liberated from its phosphate ester after incubation with alkaline phosphatase at 37° and pH 9·2, as shown by the presence of orcinol-reacting material in filtrates obtained after treatment with the $Ba(OH)_2$–$ZnSO_4$ reagents of Somogyi (1945). In view of the small amount of material available, polarimetric examination was not feasible, but paper chromatography strongly

Table 1. *Examination of the biosynthetic pentose phosphate for contamination with sedoheptulose phosphate*

Samples (about 0·2 μmole) of the barium salts of the biosynthetic pentose phosphate and of synthetic ribose 5-phosphate were subjected to the orcinol reaction and optical densities measured at 580 and 670 mμ. in a Unicam spectrophotometer.

	Amount taken (μmole)	Optical density at (*a*) 580 mμ.	(*b*) 670 mμ.	Ratio *b*/*a*
Synthetic ribose 5-phosphate	0·146	0·101	0·379	3·76
Biosynthetic pentose phosphate	0·218	0·158	0·590	3·74

suggested that the pentose was in fact ribose. When a chromatogram was run with phenol as solvent (Partridge, 1948), followed by spraying with aniline hydrogen phthalate, the pentose was readily separated from admixture with D-arabinose or D-xylose, but travelled as a single spot when mixed with authentic D-ribose.

This conclusion was confirmed by making use of the known specificity of the ribokinase present in extracts of ribose-grown *Esch. coli*. As shown in the previous paper (Heald & Long, 1955), these bacterial extracts will utilize D-ribose when supplemented with ATP and magnesium ions, but have no effect on D-xylose or D- or L-arabinose. When the biosynthetic pentose phosphate was incubated first with alkaline phosphatase and then, after inactivating this enzyme, with the ribokinase and ATP, the free pentose formed by the action of the first enzyme was found to be well utilized by the second. This experiment (Table 2) also strongly suggests that the pentose phosphate is in fact a ribose phosphate. The similar results, obtained in a parallel experiment with a sample of synthetic ribose 5-phosphate, are also shown for comparison in Table 2. It should, of course, be pointed out that this experiment does not exclude the possibility that the pentose may be ribulose, which perhaps is also a substrate for ribokinase; however, as shown in the next section, there is strong evidence against the presence of ketopentose.

Examination of the biosynthetic product for ketose. Since any sugar containing a potential aldehyde group may be oxidized by bromine water to the corresponding carboxylic acid, whereas ketoses and their derivatives are not oxidizable under these conditions, one may readily determine the proportions of aldose and ketose derivatives in a mixture. In the pentose series, both aldoses and ketoses give a positive orcinol reaction, but the carboxylic acid formed by oxidation of an aldopentose will not react with the reagent. It follows, then, that bromine oxidation of ribose phosphates (except ribose 1-phosphate) will result in material giving a negative orcinol reaction, whereas bromine treatment will not affect the ribulose phosphates. The action of bromine water, in the presence of magnesium carbonate, has been applied to the biosynthetic pentose phosphate and to the other ribose phosphates, with the results shown in Table 3.

Table 2. *The nature of the pentose in the biosynthetic pentose phosphate*

Authentic D-ribose or the pentose solutions (about 0·6 μmole), obtained by the action of rabbit intestinal mucosa alkaline phosphatase on synthetic ribose 5-phosphate or the biosynthetic pentose phosphate (see Methods section), were incubated for 30 min. at 37° with 0·2 ml. of an extract of ribose-grown *Esch. coli* (derived from 4 mg. dry wt. of cells) in a reaction medium of the following composition: 0·005 M-ATP, 0·005 M-$MgCl_2$, 0·28 M-$(NH_4)_2SO_4$ and 0·04 M THAM buffer, pH 7·6; total vol., 0·5 ml. Reaction mixtures were deproteinized with either the $Ba(OH)_2$–$ZnSO_4$ reagents of Somogyi (1945) or with 20% (w/v) mercuric acetate in 0·1% (v/v) acetic acid (Heald & Long, 1955) and the filtrates analysed for free pentose and for free + esterified pentose, respectively. The results are given as μmoles of pentose or pentose phosphate.

	Amount present initially		Amount present at end of incubation			
Substrate	Free pentose	Pentose phosphate	Free pentose	Pentose phosphate	Pentose utilized	Pentose phosphate formed
Authentic D-ribose	0·70	0	0·29	0·53	0·41	0·53
Ribose derived from synthetic ribose 5-phosphate	0·60	0·17	0·27	0·50	0·33	0·33
Pentose derived from biosynthetic pentose phosphate	0·60	0·06	0·33	0·43	0·27	0·37

Table 3. *Bromine oxidation of pentose phosphates*

The barium salt of the pentose phosphate (about 1 μmole), dissolved in 0·5 ml. 0·01 N-HCl, was treated with about 20 mg. $MgCO_3$ and 0·25 ml. 1·2% bromine water. The mixture was incubated for 1·3 hr. at 25° and the orcinol reaction carried out directly on the suspension.

	Amount taken* for bromine oxidation (μmoles)	Amount remaining after bromine oxidation* (μmole)	Apparent ketose (%)
Biosynthetic pentose phosphate	1·09	0	0
Synthetic ribose 5-phosphate	0·73	0·10	13
Ribose 5-phosphate prepared by acid hydrolysis of ATP	0·99	0·02	2
Mixture of ribose 2- and 3-phosphates	0·93	0·02	2
Ribose	0·96	0	0

* These values are based on pentose estimation by the orcinol reaction.

These findings demonstrate that ketose derivatives were absent from the biosynthetic product, and that negligible amounts were present in the ribose 5-phosphate prepared by acid hydrolysis, or in the mixture of ribose 2- and 3-phosphates. However, about 13 % ketose appeared to be present in the specimen of synthetic ribose 5-phosphate; this value has been repeatedly confirmed, but no explanation for the result can be offered. That the latter material does not in fact contain ketopentose, however, is shown by the observation that the green solution obtained in the orcinol reaction showed no enhanced absorption in the region of 540 mμ. as compared with a ribose standard; the absorption at 540 mμ. is characteristic of ketopentoses (Horecker *et al.* 1951). Furthermore, the bromine-resistant material from the sample of synthetic ribose 5-phosphate did not show any spectrophotometric evidence for the presence of ketopentose. In fact, when examined by this spectrophotometric method, neither the biosynthetic pentose phosphate nor any of the other pentose phosphates used in this work showed any indication of contamination with ketose derivatives. These experiments, therefore, indicate that the biosynthetic pentose phosphate is a ribose phosphate.

Reducing properties of the biosynthetic ribose phosphate. Reducing power was determined with the Nelson (1944) reagent, using ribose as standard. The results for the biosynthetic ribose phosphate and for the various reference ribose phosphates are shown in Table 4. From these it will be seen that the two samples of ribose 5-phosphate have reducing powers close to that of ribose itself, and that the biosynthetic ribose phosphate is similarly strongly reducing. By contrast, the mixture of ribose 2- and 3-phosphates is only weakly reducing. These observations suggest that the biosynthetic material is ribose 5-phosphate.

Acid hydrolysis. Samples of the barium salts of the two ribose 5-phosphate samples, the mixture of ribose 2- and 3-phosphates and the biosynthetic ribose phosphate were heated with N perchloric acid on a boiling-water bath for periods up to 7 hr. The hydrolysates were then analysed for free ribose and inorganic phosphate after the different time intervals. The results are given in Table 5.

Table 4. *Reducing power of ribose phosphates*

Samples of the barium ribose phosphates (about 0·2 μmole) were quantitatively examined for reducing power by the method of Nelson (1944).

	Amount taken* (μmole)	Reducing equivalent† (μmole)	Ratio‡: reducing equivalent / ribose content
Biosynthetic ribose phosphate	0·219	0·181	0·87
Synthetic ribose 5-phosphate	0·184	0·186	1·01
Ribose 5-phosphate, prepared by acid hydrolysis of ATP	0·198	0·184	0·93
Mixture of ribose 2- and 3-phosphates	0·161	0·060	0·37

* Based on total pentose content by orcinol reaction.
† Free ribose as standard for reducing power.
‡ Ratio for free ribose is unity.

Table 5. *Acid hydrolysis of ribose phosphates*

Samples of ribose phosphates (about 1 μmole) were heated on a boiling-water bath with N-$HClO_4$ and the amounts of free ribose and inorganic phosphate were determined after different time intervals, as described in the Experimental section.

	Percentage of total liberated after (hr.)			
	1	2	4	7
Free ribose analyses*				
Biosynthetic ribose phosphate	9	—	22	32
Synthetic ribose 5-phosphate	12	20	24	31
Ribose 5-phosphate, prepared by acid hydrolysis of ATP	14	17	23	28
Mixture of ribose 2- and 3-phosphates	94	96	94	—
Inorganic phosphate analyses				
Biosynthetic ribose phosphate	27	32	54	73
Synthetic ribose 5-phosphate	20	31	56	74
Ribose 5-phosphate, prepared by acid hydrolysis of ATP	17	27	60	70
Mixture of ribose 2- and 3-phosphates	91	97	100	—

* Corrected for decomposition of ribose, when heated in N acid. For explanation, see text.

It was found in a parallel control experiment with ribose that, under the conditions of acid hydrolysis used here, the extent of its decomposition, as measured by the orcinol reaction, amounted to 4, 6, 7 and 15% after 1, 2, 4 and 7 hr., respectively. The values for free ribose in Table 5 have therefore been corrected for this slight decomposition, although it is clear that the correction should be rather smaller, since there is no free ribose at zero time.

It will be observed that the liberation of free ribose and inorganic phosphate from the mixture of ribose 2- and 3-phosphates is more than 90% complete after 1 hr., whereas in the case of the two ribose 5-phosphate samples only 12–14% of the ribose and 17–20% of the phosphate appear to be liberated during this period. It is not clear why the rates of liberation of ribose and inorganic phosphate do not run parallel during the hydrolysis of ribose 5-phosphate, but the very considerable discrepancy is evident after all time intervals, the ratio pentose:inorganic phosphate averaging about 0·4. A possible explanation of this behaviour is that prolonged treatment with hot mineral acid first converts some of the ribose 5-phosphate into an unknown phosphorylated intermediate, and that when this subsequently undergoes hydrolysis, inorganic phosphate is formed together with a degradation product of ribose which does not give a positive orcinol reaction.

The rate of hydrolysis of the biosynthetic ribose phosphate is clearly very similar to that of the two ribose 5-phosphate samples, and quite different from that of the ribose 2- and 3-phosphate mixture. The fact that the authentic ribose 5-phosphate and the biosynthetic ribose phosphate both show the discrepancy between the rates of ribose and phosphate liberation gives added support to the likelihood that they are identical.

Periodate oxidation. Ribose 5-phosphate should react with 3 molecular proportions of periodic acid, whereas both ribose 2- and 3-phosphates should react with only two. When the barium salts of the biosynthetic ribose phosphate and of the other ribose phosphates were treated with about a twofold excess of periodic acid for 30–120 min., the results in Table 6 were obtained. It will be seen that the ribose 5-phosphate samples gave, within experimental error, the theoretical value for the molar ratio, periodic acid:ribose. The biosynthetic ribose phosphate also required about 3 moles periodic acid/mole ribose. The mixture of ribose 2- and 3-phosphates gave rather lower than the theoretical value. This experiment supports the view that the biosynthetic material is ribose 5-phosphate.

Further evidence for this conclusion was obtained by determining the formaldehyde production after treatment with periodate, using the procedure of O'Dea & Gibbons (1953). It was found that yields of 0·10 and 0·08 mole of formaldehyde were obtained from 1 mole of biosynthetic ribose phosphate and synthetic ribose 5-phosphate, respectively. It can be inferred from these results that the—CH_2OH group is not free in position 5 in the biosynthetic material, for otherwise the yield of formaldehyde would have been 1·0 mole/mole ribose phosphate.

Rate of colour development in the orcinol reaction. The rate at which the green colour develops in the orcinol reaction for pentoses has long been known to depend on the structure of the pentose derivative. Thus, pentoses with a phosphate substituent in C_5, as in ATP, ADP, AMP and ribose 5-phosphate, react more readily than do the pentoses themselves or their derivatives with phosphate substituents in C_2 or C_3. In the present work, samples of the ribose phosphates (about 0·2 μmole) were heated for different periods with the orcinol reagent and the extent of colour development, expressed as a percentage of that found after 40 min., was determined.

Table 6. *Periodate oxidation of ribose phosphates*

The barium salt of each ribose phosphate (about 1 μmole), dissolved in 0·5 ml. 0·01 N-HCl, was treated with 1 ml. approx. 0·0067 M-HIO_4 (in 0·1 M acetate buffer, pH 5·0). After 30, 60 or 120 min., 1 ml. 2% (w/v) KI in 4·5% (w/v) $NaHCO_3$ and one drop sat. aqueous Na_2SO_4 were added, and the mixture titrated with standard 0·1 N arsenite from a Conway burette, with one drop of starch indicator added near the end point. Controls with water instead of ribose phosphate, at the same time intervals, and with ribose phosphates added after the HIO_4, KI, $NaHCO_3$ and Na_2SO_4 were simultaneously carried out. The values given are corrected for these controls. Since there were no differences in the titration readings obtained after 30, 60 and 120 min. in the experimental tubes, the figures have been averaged.

	Amount* taken (μmole)	0·1 N arsenite required (ml.)	Molar ratio, HIO_4:ribose	
			Obs.	Calc.
Synthetic ribose 5-phosphate	0·824	0·0504	3·05	3·0
Ribose 5-phosphate, prepared by acid hydrolysis of ATP	0·794	0·0473	2·97	3·0
Mixture of ribose 2- and 3-phosphates	0·931	0·0300	1·61	2·0
Biosynthetic ribose phosphate	0·750	0·0471	3·14	—

* Based on pentose content by orcinol determination.

The results are represented graphically in Fig. 1. It will be seen that ribose and the mixture of ribose 2- and 3-phosphates reacted relatively slowly with the reagent, whereas the two ribose 5-phosphate samples and the biosynthetic ribose phosphate all reacted much more rapidly. These results also confirm the view that the biosynthetic product is ribose 5-phosphate.

DISCUSSION

Although the biosynthetic pentose phosphate isolated in this work has not been obtained completely pure, the evidence strongly favours the structure ribose 5-phosphate being assigned to it. The barium salt after purification contained no material absorbing at 260 mμ., indicating its freedom from contamination by the adenosine triphosphate used in its preparation. It was also quite free from inorganic phosphate and had a pentose:phosphorus ratio of 0·86. Furthermore, it was uncontaminated by heptulose phosphate, as shown spectrophotometrically on the green solution formed in the orcinol reaction (Table 1), nor did it contain any ketopentose derivative, as indicated by oxidation studies with bromine water (Table 3). The pentose was almost certainly ribose, for alkaline phosphatase liberated a free pentose, which could be rephosphorylated by a ribokinase preparation in the presence of adenosine triphosphate. It has already been shown in the previous paper (Heald & Long, 1955) that D-xylose and D- and L-arabinose were not enzymically phosphorylated under these conditions, whereas D-ribose reacted readily.

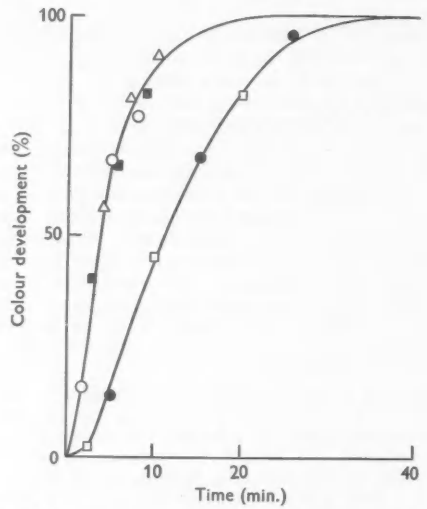

Fig. 1. Rates of colour development with ribose and with different ribose phosphates in the orcinol reaction. Ribose phosphate (about 0·2 μmole) heated with the orcinol reagent on a boiling-water bath for various periods. Colour intensity measured in a photoelectric colorimeter and expressed as a percentage of the maximum obtainable at 40 min. □, ribose; ●, mixture of ribose 2- and 3-phosphates; ○, biosynthetic ribose phosphate; ■, synthetic ribose 5-phosphate; △, ribose 5-phosphate, prepared by acid hydrolysis of ATP.

Evidence for the position of the phosphate group at C_5 is equally convincing. Thus, the stability in N acid at 100° of ribose 5-phosphate is much greater than that of the mixture of ribose 2- and 3-phosphates (Table 5), and the biosynthetic ribose phosphate had similar stability to that of the ribose 5-phosphate samples. Ribose 1-phosphate, of course, is excluded because of its very great acid lability (Lowry & Lopez, 1946). The action of periodic acid strongly supports the ribose 5-phosphate structure (Table 6), one molecule of the biosynthetic product reacting with but slightly more than the three molecules of periodic acid required by theory. The rate of colour development with the orcinol reagent (Fig. 1) is also consistent with this conclusion. Finally, the reducing power accords well with the ribose 5-phosphate structure (Table 4), the latter being a much stronger reducing agent under the conditions employed than the mixture of ribose 2- and 3-phosphates, although the reason for this difference is obscure. The reducing properties also effectively rule out the possibility of the material being ribose 1-phosphate, which is non-reducing under alkaline conditions.

SUMMARY

1. Large-scale enzymic phosphorylations of D-ribose, by means of adenosine triphosphate in the presence of a cell-free extract of ribose-grown *Escherichia coli*, have been carried out. A biosynthetic pentose monophosphate has been isolated from the reaction mixture and partially purified.

2. This biosynthetic material is almost certainly the barium salt of ribose 5-phosphate, as shown by the liberation of ribose after the action of alkaline phosphatase, and by studies of reducing power, acid stability, periodate oxidation and rate of colour development in the orcinol reaction.

I am indebted to Miss Iris Creighton for skilled technical assistance.

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The Occurrence of a Cyanocobalamin-binding Protein in Milk and the Isolation of a Cyanocobalamin–Protein Complex from Sow's Milk

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A preliminary communication from this laboratory has described the occurrence of a vitamin B_{12}-binding substance in sow's milk whey (Gregory, Ford & Kon, 1952). More recently, we have shown that this binding substance, and an intrinsic-factor concentrate prepared from pig stomach, combined with other vitamin B_{12}-like substances in the same way as with cyanocobalamin (vitamin B_{12}) (Gregory & Holdsworth, 1953). The similarity between the effect on cyanocobalamin of the sow's milk whey and of the intrinsic-factor concentrates, led us to attempt the isolation of the binding substance from sow's milk, since a study of its properties may help to explain some aspects of the utilization and metabolism of cyanocobalamin.

The first part of this paper presents evidence that the milk of the cow, goat, pig, rat and woman possesses the property of combining with added cyanocobalamin. The second part describes the isolation from sow's milk of a cyanocobalamin–protein complex.

THE OCCURRENCE OF A CYANOCOBALAMIN-BINDING PROTEIN IN MILK

Experimental

Materials

The samples of milk from the cow, goat, pig, rat and woman and of colostrum from the cow and goat were the same as those described by Gregory (1954).

The sow's whey concentrate (SF 8) and the 'intrinsic factor' concentrate from pig stomach were prepared as described by Gregory & Holdsworth (1953).

Microbiological methods

Method of assay. The method of assay with *Lactobacillus leichmannii* ATCC 4797 was a modification of that of Skeggs, Nepple, Valentik, Huff & Wright (1950) and is fully described by Gregory (1954).

Measurement of cyanocobalamin in the cyanocobalamin–protein complex. Cyanocobalamin was quantitatively released from the protein complex by digestion with papain as described by Gregory (1954) and measured by *Lb. leichmannii*.

Measurement of cyanocobalamin-binding activity. Two methods were used. (*a*) The growth-inhibition method, described by Gregory & Holdsworth (1953). (*b*) The ultrafiltration method. A bag of 'Visking' cellulose tubing (The Visking Corporation, Chicago), diameter $\frac{1}{2}$ in., was suspended from the stem of a glass funnel held in the neck of a filtration tube by means of a rubber bung. The bag was made by knotting one end of the tubing tightly and tying the other, with cotton, over a piece of Polythene tubing fitted over the stem of the funnel. (A diagram of this apparatus will be found in the paper by Gregory, 1954.) A known amount of cyanocobalamin in 1 ml. of solution was added to a measured amount of the binding substance, and the mixture (total volume 10 ml.) poured into the cellophan bag. The filtration tube was evacuated, sealed off and left in the refrigerator for 1–2 hr. In this time, 1–2 ml. of ultrafiltrate could be collected.

The free cyanocobalamin, which was able to pass into the ultrafiltrate, was measured by *Lb. leichmannii*. The amount of cyanocobalamin bound by the binding substance was then calculated by subtracting the free cyanocobalamin from the total amount added.

This method was applied to the measurement of the binding of cyanocobalamin by the milks of different animal species. The approximate amount of cyanocobalamin bound by each of the milks was first estimated and then a more exact determination made. Three different levels of cyanocobalamin within a narrow range (e.g. 0·05, 0·10 and 0·20 μg.) were added to 10 ml. milk, so that at least two of the levels gave an estimation of the binding activity.

Comparison of the bound form of cyanocobalamin in the milk of different species and in extracts of desiccated pig stomach

In order to establish whether the cyanocobalamin in the different milks was associated with one particular protein, the whey proteins were separated by electrophoresis on paper and the paper strips examined for proteins and

cyanocobalamin by the methods now to be described. An 'intrinsic factor' concentrate, prepared from desiccated pig stomach as described by Gregory & Holdsworth (1953), was treated similarly.

Preparation of the samples for electrophoresis

The milk was centrifuged and the cream removed and discarded. Glacial acetic acid was added to the skimmed milk to bring the pH to 4·6. The precipitated casein was removed by centrifuging and the precipitate washed once with 0·1 M sodium acetate buffer at pH 4·6. The combined whey and washings were dialysed against running tap water for 6 hr., then against 0·05 M veronal buffer at pH 8·6 for 16 hr. The whey was then concentrated by ultrafiltration (using the apparatus described above), and adjusted to a final concentration of 2% protein (estimated by the biuret reaction of Levin & Brauer, 1951). The 'intrinsic factor' concentrate and the sow's whey concentrate (SF 8) were each used as 2% solutions in 0·05 M sodium veronal buffer, pH 8·6.

Protein patterns. A band of each protein solution 5 cm. long and 0·3 cm. wide was painted on to a wide strip of Whatman no. 3 MM paper and separated by electrophoresis in 0·05 M sodium veronal buffer at pH 8·6 under a potential gradient of 7 v/cm. Under these conditions a marker spot of 2% crystalline bovine albumin moved 16 cm. in 15 hr. In addition to the whey proteins a spot of 1% lactose solution was added to each strip to mark the extent of electro-osmosis.

The separated proteins on the paper strips were stained with bromophenol blue as recommended by Kunkel & Tiselius (1951) and the strips were scanned for the stained protein at 2 mm. intervals in a densitometer (made by E. S. Holdsworth, unpublished).

Electrophoresis followed by microbiological assay. A piece of Whatman no. 3 MM paper was marked in 1 cm. strips at right angles to the electrical field, and 0·1 ml. of the protein solution was applied at the origin as a row of 0·01 ml. spots 1 cm. apart. When the electrophoresis in 0·05 M sodium veronal buffer had been completed, the wet paper was immediately cut into the 1 cm. strips. Each strip was rolled up and placed on a 3 cm. square of fine nylon stocking fabric, which was arranged in the mouth of a ½ oz. screw-capped bottle so that its edges were overhanging. The cap was screwed on to the bottle tightly, making, in effect, a nylon bag in which the paper strip was suspended half-way down the bottle. The bottles were centrifuged at 2000 *g* for 15 min. and liquid from the paper strips collected in the bottom of the bottles. The papers were moistened with physiological saline and centrifuged again. In this way the protein was washed from the paper strips. These protein solutions were made to known volumes and portions removed for microbiological assay either for total cyanocobalamin or for binding activity measured by the growth-inhibition method.

Autoradiography. In some instances, e.g. with the sample of sow's milk and the 'intrinsic factor' and sow's whey (SF 8) concentrates, radioactive cyanocobalamin labelled with ^{60}Co was added before electrophoresis on paper. The position of material containing radioactive cyanocobalamin was shown by placing the dry electrophoresis strips in contact with Ilfex (Ilford Ltd.) X-ray film. After the required exposure, the film was developed according to the maker's instructions. This was a sensitive test for cyanocobalamin, e.g. 0·01 ml. of a solution containing 0·005 μg. cyanocobalamin/ml. (activity 420 μc/mg.) gave on the film,

Table 1. *Measurement of the 'binding' of cyanocobalamin by the milks of different species of animals, and of the cyanocobalamin naturally present in these milks*

Naturally occurring cyanocobalamin was measured after papain digestion by the method of Gregory (1954); cyanocobalamin-binding activity was measured by the ultrafiltration method described in the text: assay organism: *Lb. leichmannii*. Means in bold type.

Material	Amount of cyanocobalamin added/ml. milk (μg.)	Amount of cyanocobalamin found/ml. of ultrafiltrate (μg.)	Cyanocobalamin 'bound' (μg./ml. milk)		Natural cyanocobalamin present (μg./ml. milk)
Sow's milk	0·200	0·000	>0·200		
	0·300	0·070	0·230	**0·245**	0·0017
	0·400	0·140	0·260		
Rat's milk (diluted 1:30)	0·0004	0·000			
	0·004	0·0006	0·102		
	0·050	0·0450	0·150	**0·126**	0·0120
Human milk	0·050	0·000	>0·050		
	0·100	0·020	0·080	**0·080**	0·0003
	0·200	0·120	0·080		
Cow's colostrum	0·008	0·0000	>0·008		
	0·010	0·0018	0·0082	**0·0083**	0·0029
	0·0125	0·0042	0·0083		
Goat's colostrum	0·008	0·0023	0·0057		
	0·010	0·0044	0·0056	**0·0058**	0·0050
	0·0125	0·0064	0·0061		
Goat's milk	0·004	0·0014	0·0026		
	0·006	0·0035	0·0025	**0·0027**	0·0007
	0·008	0·0050	0·0030		
Cow's milk	0·002	0·0017	0·0003		
	0·004	0·0037	0·0003	**0·0005**	0·0032
	0·006	0·0051	0·0009		

an easily discernible zone after an exposure of 7 days. An accurate measure of the darkening of the film due to the radioactivity was obtained by scanning the film at 2 mm. intervals in the densitometer used for examining protein patterns.

Liquid counting. Accurate measurement of radioactivity was unnecessary for the protein fractionation procedures. A McMichael radiation monitor was used as a rate meter by immersing the graphite-electrode counter to a standard depth in a shielded measuring cylinder containing the solution to be assayed. The activity of each fraction was expressed as a percentage of the activity present before the beginning of the fractionation.

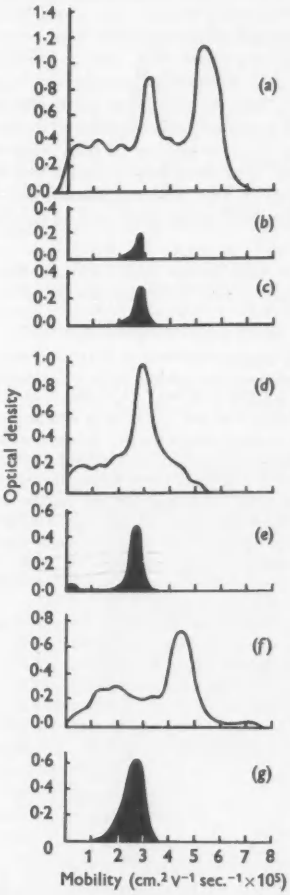

Fig. 1. Electrophoretic patterns of milk wheys. Electrophoresis in sodium veronal buffer, pH 8·6. The blocked-in curves represent cyanocobalamin measured microbiologically, the unblocked curves represent protein distribution. The ordinates are arbitrary units (optical density) of protein or cyanocobalamin concentrations: (*a*) cow's whey proteins, (*b*) cow's whey natural cyanocobalamin, (*c*) cow's whey with added cyanocobalamin, (*d*) goat's whey proteins, (*e*) goat's whey with added cyanocobalamin, (*f*) human whey proteins, (*g*) human whey with added cyanocobalamin.

Results

Cyanocobalamin-binding activity of the milks of different animal species. The addition of cyanocobalamin to the different milks resulted in some of the vitamin being bound to protein and therefore no longer able to pass through a cellophan membrane. It can be seen from Table 1 that each of the milks bound a definite amount of cyanocobalamin, and that cyanocobalamin added over and above this amount was recovered in the ultrafiltrate. A solution of the pure vitamin could be ultrafiltered quantitatively.

The bound form of cyanocobalamin in the different milks. The whey proteins from each of the milks were separated by electrophoresis on paper as described in the experimental section. Figs. 1 and 2

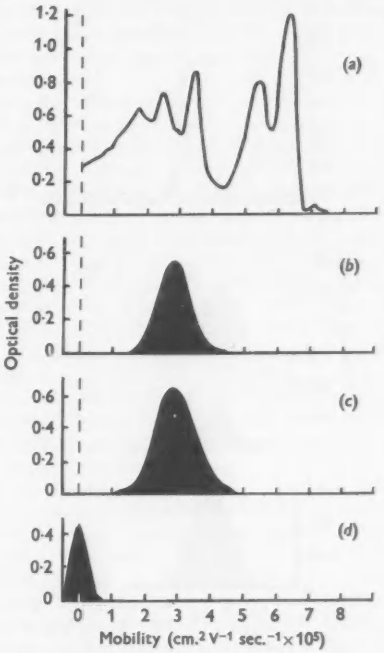

Fig. 2. Electrophoretic patterns of sow's milk whey and an 'intrinsic factor' concentrate. Electrophoresis in sodium veronal buffer, at pH 8·6. The blocked-in curves represent added cyanocobalamin (^{60}Co) measured as the optical density of the autoradiographs: (*a*) sow's whey proteins, (*b*) sow's whey with added cyanocobalamin, (*c*) 'intrinsic factor' concentrate with added cyanocobalamin, (*d*) cyanocobalamin control.

illustrate the results of analysing the paper strips, after electrophoresis, for the position of the proteins (stained by bromophenol blue) and of the cyanocobalamin (measured by *Lb. leichmannii* or by autoradiography).

Owing to the low natural content of cyanocobalamin in goat's and human milks, the wheys from these milks were saturated with cyanocobalamin before electrophoresis (using the figures shown in Table 1), so that the position of the bound vitamin could be measured with more accuracy. Cyanocobalamin added to cow's whey moved to the same position as that naturally present in the whey (Fig. 1) and furthermore the bound cyanocobalamin had the same mobility in cow's, goat's, human and sow's wheys, although the protein patterns were dissimilar. In addition, Fig. 2 shows that cyanocobalamin bound by extracts of desiccated pig stomach (the 'intrinsic factor' concentrate) had the same mobility as the bound form in milk. Under the experimental conditions, cyanocobalamin itself is uncharged and has zero mobility.

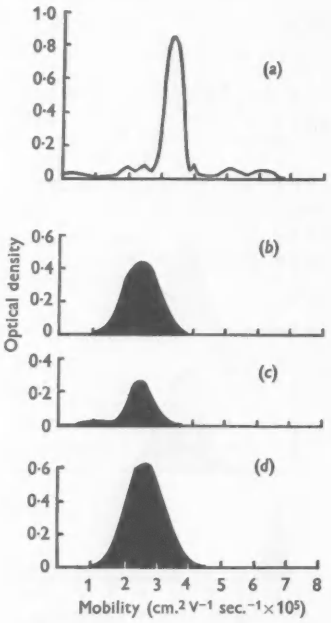

Fig. 3. Electrophoretic patterns of the sow's whey protein fraction SF 8. Electrophoresis in veronal buffer pH 8·6. The ordinates are arbitrary units of protein or cyanocobalamin concentration or of cyanocobalamin-binding activity: (*a*) SF 8 protein pattern, (*b*) cyanocobalamin-binding activity of SF 8, (*c*) cyanocobalamin naturally present in SF 8, (*d*) SF 8 with added cyanocobalamin (^{60}Co).

The bound form of cyanocobalamin in sow's milk. Examination of the protein patterns of the sow's whey protein concentrate (SF 8), which contained the bulk of the cyanocobalamin-binding activity of the whey, revealed only one major protein constituent, lactalbumin (see Fig. 3*a*). The binding substance had a mobility slightly less than the lactalbumin (Fig. 3*b*). Fig. 3*b–d* shows that the binding substance, the cyanocobalamin–protein complex occurring naturally in the whey, and the complex formed by saturating the whey with added cyanocobalamin, all had the same electrophoretic mobility. The similarity between the bound form of cyanocobalamin occurring naturally and that produced by adding cyanocobalamin to the milk led us to adopt the procedure of fully saturating the whey with cyanocobalamin in order to obtain larger yields of the cyanocobalamin–protein complex as described in the next part of this paper.

THE ISOLATION OF A CYANOCOBALAMIN–PROTEIN COMPLEX FROM SOW'S MILK

Experimental and Results

The binding substance present in 2700 ml. of skimmed milk was fully saturated by adding 540 μg. radioactive cyanocobalamin, labelled with ^{60}Co. The activity of this material was 0·24 μc/mg. The pH of the milk was adjusted to 4·6 with glacial acetic acid and the precipitated casein removed by centrifuging. The casein was washed twice with 0·1 M sodium acetate buffer at pH 4·6, and the washings were added to the whey. The combined washings and whey were saturated to 33% with $(NH_4)_2SO_4$. The precipitated proteins were removed by centrifuging and washed with a 33% saturated $(NH_4)_2SO_4$ solution. Since the precipitate contained no radioactivity, measured in the liquid counter, it was discarded.

The whey and washings were fully saturated with $(NH_4)_2SO_4$. The precipitate was removed and suspended in 300 ml. 0·1 M sodium acetate buffer at pH 5·5 containing a few mg. of cysteine. The insoluble material was removed by centrifuging and discarded, since it had no radioactivity. The supernatant fluid was treated with saturated ammonium sulphate solution and the following fractions were collected.

Fraction	Saturation with $(NH_4)_2SO_4$ (%)	Radioactivity (%)
A	0–33	0
B	33–50	0
C	50–60	15
D	60–66	15
E	66–100	70

Each of the fractions *C*, *D* and *E* was dissolved in 30 ml. water and dialysed for 6 hr. against dilute sodium acetate buffer containing cysteine. Fraction *E* was freeze-dried and 1 g. of a pink powder containing 520 μg. cyanocobalamin obtained. The powder was dissolved in 20 ml. 0·025 M sodium veronal buffer at pH 8·6 and separated by continuous electrophoresis on paper (Holdsworth, 1953). Whatman no. 54 paper, with sodium veronal buffer at

pH 8·6 (0·025 M) and a potential of 900 V were used; Fig. 4 shows the paper at the end of the experiment after staining with bromophenol blue. Tubes 9 and 10 contained the bulk of the cyanocobalamin, measured both microbiologically and by the radioactivity, and thus good separation from the bulk of the proteins present was obtained.

The contents of the tubes with the bound cyanocobalamin were combined, and concentrated by ultrafiltration to a volume of 5 ml. This concentrated solution at pH 8·6 was fractionated with *iso*propanol at −5°.

Precipitate	*iso*Propanol (%)	Radioactivity (%)
1	0–28	5
2	28–45	90
3	45–55	5
4	55–75	0

Precipitate 2 was suspended in 2 ml. 0·1 M sodium acetate buffer at pH 4·8. The insoluble material was removed, washed with buffer and discarded, as it had no radioactivity. The supernatant fluid and the washings were refractionated at −5° with *iso*propanol. The precipitate obtained at 45–55% *iso*propanol was red. This red precipitate was purified by further fractionations with *iso*propanol at pH 4·8 until the ratio of the extinction coefficients at 278 (due to protein) and 362 mμ. (due to cyanocobalamin) remained at a steady value.

In the final purification procedure, where the quantity of material handled was very small, the main object was to obtain a pure material and the overall yield was of secondary importance. A 45-fold concentration of the cyanocobalamin–protein complex, present in 1 g. of crude material (fraction *E*) was achieved. The pink powder, finally obtained, weighed 3·3 mg. and contained 78 μg. cyanocobalamin (calculated from the absorption at 362 mμ.), that is 23·6 μg./mg. of the complex. It was readily soluble in water and soluble in 50% (v/v) ethanol.

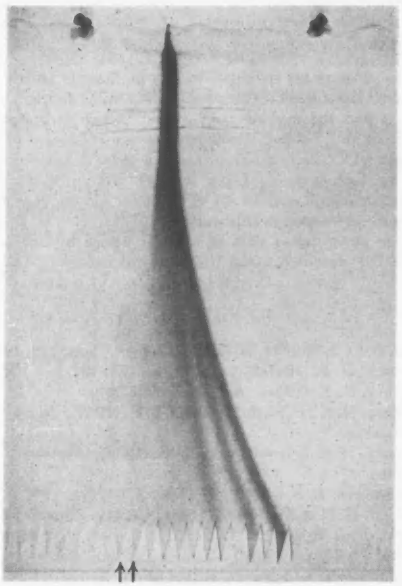

Fig. 4. Photograph showing the separation of the sow's whey proteins after continuous electrophoresis on paper. The arrows mark the position of the cyanocobalamin–protein complex (tubes 9 and 10).

The amount of material obtained was insufficient to apply the usual criteria for protein purity. Therefore the assessment of purity was based on the fact that the substance was homogenous when examined by electrophoresis on paper at both pH 6·5 (phosphate buffer) and pH 8·6 (veronal buffer), and that the ratio of the concentration of cyanocobalamin to protein was unaltered by further precipitation with *iso*propanol.

DISCUSSION

Gregory *et al.* (1952) found that the cyanocobalamin of sow's milk could not be measured by a procedure which gave good results with cow's milk (see also Gregory, 1954). They found further that cyanocobalamin, added to the milk to a concentration of 0·06 μg./ml. was unavailable to the assay organism, which responded, however, to additions over and above that amount. In other words, sow's milk could inactivate 0·06 μg. cyanocobalamin/ml. This figure was obtained after heating the diluted whole milk with the assay medium and cyanocobalamin. In a previous paper (Gregory & Holdsworth, 1953) we have shown that an unheated protein concentrate from sow's milk bound more cyanocobalamin than the heated concentrate. It was necessary, therefore, to devise some method for measuring the amount of cyanocobalamin bound by unheated whole milk. Natural inhibitors present in raw milk complicate the interpretation of the microbiological responses when unheated milk was added aseptically to the assay tubes (Gregory, 1954).

In the ultrafiltration method finally adopted, the actual binding process was separated from the microbiological assay. The technique consisted of mixing the milk or binding protein with a slight excess of cyanocobalamin, and ultrafiltering the mixture. By removing only a small proportion of the fluid by ultrafiltration, the equilibrium of the system was not appreciably disturbed and the concentration of cyanocobalamin measured in the ultrafiltrate represented the concentration of the free vitamin in the whole system. (The Donnan effect for neutral cyanocobalamin is negligible.) When the amount of cyanocobalamin bound by a sow's whey concentrate was measured by this ultrafiltration method, the result was identical with that obtained

previously by a growth-inhibition method (Gregory & Holdsworth, 1953). The use of ultrafiltration seems more justifiable than the techniques depending on dialysing the protein–cyanocobalamin mixture for long periods against large volumes of buffer or saline as used by Bird & Hoevet (1951) and Rosenthal & Sarett (1952). This improved method has shown that sow's milk can combine with 0·24 μg. cyanocobalamin/ml. (see Table 1), a figure 4 times that obtained when the milk was heated with the basal medium for microbiological assay (Gregory *et al.* 1952). It has also shown that the milk and colostrum of other animal species have the capacity of binding additional cyanocobalamin, a property not detected when the milks were heated with the basal assay medium.

The wheys from the various milks showed different protein patterns when examined by electrophoresis on paper (Figs. 1 and 2), in good agreement with results obtained by Deutsch (1947) for electrophoresis in free solution using the conventional Tiselius apparatus. Microbiological assays and autoradiographs of milk extracts, after separation by electrophoresis on paper showed that the bound form of the vitamin in the milks, and the complexes formed when the vitamin was added to the milks, were very similar if not identical. Moreover, the bound form of cyanocobalamin from milk had the same mobility as the cyanocobalamin–protein complex isolated from desiccated pig stomach. The different heat stability of the complex in these milks makes it impossible to state that the milks all contain the same bound form of cyanocobalamin. For example, the cyanocobalamin in cow's milk is readily available to *Lb. leichmannii* after heating with the basal assay medium, whereas the cyanocobalamin in sow's milk is not released by this treatment (Gregory, 1954). We have found (Gregory & Holdsworth, 1953) that the heat-stability of the complex from sow's milk depended on the composition of the assay medium and therefore the composition of the different milks may have some influence on the stability of the complex.

Earlier experiments showed that the substance responsible for inactivating cyanocobalamin was not one of the major components of sow's whey (Fig. 3). Attempts to isolate the binding factor from sow's whey were unsuccessful because of considerable losses during fractionation and a more satisfactory procedure was to saturate the binding factor with radioactive cyanocobalamin and isolate the more stable complex by following the radioactivity.

We have shown that cyanocobalamin occurs in a bound form in milk from different animal species. The addition of cyanocobalamin to desiccated pig stomach or to human gastric juice produces a similar bound form of the vitamin. Whatever the mechanism by which Castle's intrinsic factor causes the absorption of cyanocobalamin, it seems certain that the protein-bound vitamin is implicated.

The chemical nature of the complex, and studies on the mode of linkage between its two components, are described in the following paper.

SUMMARY

1. An ultrafiltration method has been used to demonstrate that the milks of different animal species have the property of combining with added cyanocobalamin. The amount of the vitamin bound per ml. of milk was: pig, 0·24 μg.; rat, 0·13 μg.; woman, 0·08 μg.; goat, 0·0027 μg.; and cow, 0·0005 μg.

2. The whey proteins of each of the milks were separated by electrophoresis on paper and the position of the proteins on the paper compared with the position in which the cyanocobalamin occurred. In this way it was shown that the major proteins of the wheys did not combine with the vitamin and that the cyanocobalamin–protein complex had the same mobility in each of the milks tested.

3. The isolation of the pure cyanocobalamin–protein complex from sow's milk whey was achieved by fractional precipitation with ammonium sulphate, separation from the bulk of the proteins by continuous electrophoresis on paper, and finally fractional precipitation with *iso*propanol.

The authors are indebted to Drs R. Braude and K. G. Mitchell for the collection of the sow's milk, and to Dr D. Morris and the nursing staff of the British Hospital for Mothers and Babies, Woolwich, for the collection of the human milk. Their thanks are also due to Dr E. Lester Smith (Glaxo Laboratories, Ltd.) for the gift of radioactive cyanocobalamin and to Dr S. K. Kon for his continued advice and interest in this work.

This paper forms part of a Ph.D. thesis by one of us (M.E.G.) presented to the University of Reading.

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Some Properties of the Cyanocobalamin–Protein Complex from Sow's Milk, and the Mode of Linkage of Cyanocobalamin with Protein

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The occurrence of a cyanocobalamin-binding protein in milk and the isolation of a cyanocobalamin–protein complex from sow's milk whey is described in the preceding paper (Gregory & Holdsworth, 1955). The first part of this paper describes some chemical and microbiological properties of the complex, and provides evidence that it is a combination of cyanocobalamin with a glycoprotein. The manner in which these two components are linked together has been investigated in two ways. One was to test whether blocking specific groups on the protein decreased its capacity to combine with cyanocobalamin. The second was to modify the cyanocobalamin molecule and test whether it still combined with unaltered protein. The results of these investigations are presented in the second part of this paper.

SOME PROPERTIES OF THE CYANOCOBALAMIN–PROTEIN COMPLEX

Experimental and results

Absorption spectrum. The material, isolated as described in the preceding paper (Gregory & Holdsworth, 1955), was examined in aqueous solution (0·5 mg./ml.) for absorption in the visible and u.v. regions, using a Beckman Spectrophotometer.

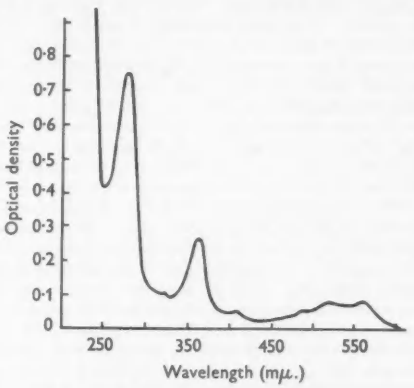

Fig. 1. The absorption spectrum of the cyanocobalamin–protein complex in aqueous solution (0·5 mg./ml.).

The curve (Fig. 1) shows pronounced peaks at 278 and 362 mμ. and inflexions at 410, 520 and 550 mμ., these last four bands arising from absorption due to the cyanocobalamin molecule. The slight shift of the peak from 361 mμ., as in free cyanocobalamin, to 362 mμ. in the complex, was repeatedly observed and is regarded as significant. Considerable excess cyanide ion at pH values up to 11 did not alter the position of the 362 mμ. band of the complex. If it is assumed that combination with protein has not affected the height of the absorption band at 361–362 mμ. of cyanocobalamin ($E^{1\%}_{1\,cm.}=204$), the optical density of the solution of the complex at 362 mμ., $E^{1\%}_{1\,cm.}=5{\cdot}7$, can be used to calculate its cyanocobalamin content. From the data obtained 1 mg. of the complex contains 23·6 μg. cyanocobalamin. The absorption band at 278 mμ., $E^{1\%}_{1\,cm.}=15{\cdot}1$ is almost entirely due to the protein component.

Estimation of tryptophan and tyrosine. The tryptophan and tyrosine content of the complex was measured by the method of Holiday (1936), which compares the optical densities, at 280 and 305 mμ., of the material dissolved in 0·1 N-NaOH. For a solution containing 0·5 mg. complex/ml. of 0·1 N-NaOH, $E_{280}=1{\cdot}26$ and $E_{305}=0{\cdot}66$, and from the formulas given by Holiday the tyrosine content was calculated as 17·6% and that of tryptophan as 2·7%.

The value 17·6% is unusually high for the tyrosine content of a protein and a control experiment with a protein known to have a high tyrosine content was done under the same conditions. We found 13% tyrosine in crystalline pepsin (Armour), a figure higher than the generally accepted value of 10% (Calvery, Herriott & Northrop, 1936).

Estimation of total nitrogen. The estimation was done in triplicate with 100 μg. portions of the complex (dried over P_2O_5). The sealed-tube digestion procedure of Grunbaum, Schaffer & Kirk (1952), was used and the ammonia produced estimated by Nessler's reagent. The complex contained 16·1% N.

Amino acid composition. The complex (0·72 mg.) was dissolved in 1 ml. of a 1:1 (v/v) mixture of conc. HCl and glacial acetic acid and hydrolysed in a sealed tube at 102° for 20 hr. After evaporation *in vacuo* over KOH, the residue was dissolved in 0·1 ml. of water and examined for amino acids by two-dimensional chromatography using phenol–water and butanol–acetic acid–water, as the solvent systems. The following amino acids were detected: cystine, aspartic and glutamic acids, arginine, lysine, histidine, serine, glycine, threonine, alanine, tyrosine, valine, methionine, phenylalanine, leucine and isoleucine.

One-dimensional chromatograms were made using butanol–acetic acid–water (5:1:4 by vol.) and sprayed with reagents specific for certain amino acids. The Pauly reagent (Bolling, Sober & Block, 1949) confirmed the presence of histidine, the platinic chloride–KI reagent of Toennies &

Kolb (1951) showed cystine to be present. The Morgan & Elson method for detecting amino sugars, as adapted to paper chromatography by Partridge (1949), showed the presence of a substance with an R_F identical with that of glucosamine or chondrosamine.

Estimation of carbohydrate. A colorimetric method for estimating carbohydrate with anthrone (Morris, 1948) was used, with glucose and glycogen as reference substances. The complex contained 7·3% carbohydrate calculated as glucose. The ribose present in the cyanocobalamin molecule would not make a significant contribution to this figure.

Estimation of hexosamine. The complex (0·5 mg.) was hydrolysed with 3N-HCl at 100° in a sealed tube for 4 hr., and concentrated *in vacuo* over KOH. The residue was dissolved in 1 ml. of water and the hexosamine present estimated by the method of Elson & Morgan (1933). Albumin (0·5 mg.) and albumin (0·5 mg.) with 50 μg. glucosamine were used as negative and positive control mixtures. The complex contained 9% hexosamine calculated as glucosamine.

Microbiological estimation of cyanocobalamin in the complex. A portion of a concentrated solution of the cyanocobalamin–protein complex was diluted with sterile distilled water to contain 0·2 μg. cyanocobalamin/ml. as estimated from the absorption curve (described above). A sample (1 ml.) of this solution, added aseptically to a sterile plugged tube, was digested with 10 mg. papain (British Drug Houses Ltd.) suspended in 1 ml. of sterile 0·1M sodium acetate buffer, pH 4·6, containing a trace of cyanide. A further 1 ml. of the bound cyanocobalamin solution was digested with 2 mg. of crystalline trypsin (Armour) dissolved in 1 ml. of sterile 1% (w/v) $NaHCO_3$ solution containing a trace of calcium. The two mixtures were incubated for 3 hr. at 57°, half the enzyme being added at the beginning and the remainder half-way through the incubation period. The digests were then made to a total volume of 4 ml. with sterile distilled water. A portion of the diluted digest was removed and assayed for cyanocobalamin both unheated and heated with the assay medium. The remainder of the digest was heated for 30 min. in steam, ultrafiltered and the ultrafiltrate assayed for cyanocobalamin. For this experiment the *Escherichia coli* tube assay was used to measure cyanocobalamin as described by Gregory & Holdsworth (1953). The full availability of the cyanocobalamin for *Esch. coli*, after heating the enzyme digest of the complex with the assay medium is apparent from Table 1. The figure, 0·19 μg. of cyanocobalamin/ml. (22·4 μg./mg. substance), agrees with the 0·20 μg./ml., calculated from the optical density of the complex at 362 mμ. Although after heating of the enzyme digests the cyanocobalamin was fully available to the assay organism, only 10% of the vitamin was ultrafiltrable from this heated digest, i.e. it was not free cyanocobalamin that was being measured.

Table 1. *Liberation of cyanocobalamin from the protein complex by enzymic digestion*

The assay organism was *Escherichia coli*.

	Total cyanocobalamin assayed	
Treatment	Heated (μg./ml.)	Unheated (μg./ml.)
None	0·12	None
Papain digestion	0·19	0·04
Tryptic digestion	0·19	0·04

THE MODE OF LINKAGE OF CYANOCOBALAMIN WITH PROTEIN

Experimental

The binding factor concentrate. A protein concentrate was prepared from sow's milk whey by $(NH_4)_2SO_4$ fractionation as described by Gregory & Holdsworth (1953). This protein fraction, capable of binding 100 μg. cyanocobalamin/g., is referred to as SF 8.

Compounds related to cyanocobalamin. The monobasic acid *E*, the corresponding acid without the nucleotide, the tribasic acid, and the mixture of hexa and hepta acids were all fragments of cyanocobalamin prepared by partial hydrolysis, and were kindly supplied by Dr E. Lester Smith (Glaxo Laboratories Ltd.). The preparation of these compounds is described by Armitage *et al.* (1953). The thiocyanatocobalamin was a gift from Dr Wokes (Ovaltine Laboratories Ltd.).

The formaldehyde-treated cyanocobalamin was prepared as follows: 400 μg. cyanocobalamin were dissolved in 9 ml. of 1% (w/v) Na_2CO_3 solution, and 1 ml. of (A.R.) formaldehyde solution (*ca.* 36% (w/v) formaldehyde) was added. After standing 15 hr. at 1° the solution was evaporated to dryness *in vacuo* (temperature less than 37°) and the residue extracted with cold 80% (v/v) ethanol. The substance was purified by chromatography on paper using *sec.*-butanol saturated with water as the solvent. Only one zone, with an R_F slightly greater than that of the original vitamin, was obtained. The material was microbiologically inactive for *Esch. coli* and *Lactobacillus leichmannii* as test organisms.

Acetylation of SF 8. The method for acetylating proteins described by Baddiley, Kekwick & Thain (1952) was used. 2′-Acetylthioethylacetamide (10 mg.) was added to 5 mg. portions of SF 8 dissolved in 0·1M sodium borate buffers at pH 8·0 and 10·0. The air in the flasks containing these solutions as a shallow layer, was displaced by oxygen, the flasks were tightly stoppered and incubated at 37° for 45 hr. The reagent and buffer were then removed by dialysis and the protein solution diluted to a concentration of 1 mg./ml. Crystalline bovine serum albumin (Armour) was acetylated at pH 10 to act as control.

The effect of specific group inhibitors on the combination of SF 8 with cyanocobalamin. When 0·2 μg. cyanocobalamin was added to a solution containing 1 mg. SF 8, half the added vitamin was bound and the rest remained free. The free vitamin was estimated by the ultrafiltration method described in the preceding paper (Gregory & Holdsworth, 1955). The reagents for blocking specific groups on the protein were dissolved in 1–8·5 ml. water and added to 1 mg. SF 8 in 0·5 ml. water as shown in Table 2. The solutions were mixed well and kept at 30° for 1 hr. At the end of this time 0·2 μg. cyanocobalamin was added to each tube and the total volume made to 10 ml. The solutions were then ultrafiltered and the ultrafiltrates assayed for cyanocobalamin using *Lb. leichmannii* as described by Gregory (1954). If the amount of cyanocobalamin in the ultrafiltrate was greater than that obtained from a control containing untreated SF 8 and cyanocobalamin, then the reagent had blocked a group on the protein necessary for its combination with the vitamin. All the inhibiting reagents were tested to ascertain that they had no growth-inhibitory effect on *Lb. leichmannii* at the dilutions present in the assay tubes. The acetylated SF 8 was examined for binding activity by this method and, after saturation with [^{60}Co] cyanocobalamin,

by electrophoresis and autoradiography as described in the preceding paper (Gregory & Holdsworth, 1955).

Combination of the altered cyanocobalamin molecule with SF 8. The combination of the compounds having structures similar to cyanocobalamin with the binding protein (SF 8) was measured by the ultrafiltration method (Gregory & Holdsworth, 1955). The technique varied slightly depending on whether the substance tested possessed microbiological activity.

The monobasic acid *E* (0·2 μg.), or thiocyanatocobalamin (0·2 μg.) were added to 1 mg. SF 8 in distilled water. The volume was made to 10 ml. and the solution ultrafiltered. The ultrafiltrate containing the acid *E*, was diluted to contain 0·2 mμg. of the acid per ml., and that containing the thiocyanatocobalamin to contain 0·04 μmg. per ml. Both were assayed by *Lb. leichmannii* (Gregory, 1954). The concentrations of these two compounds were calculated from growth-response curves given by *Lb. leichmannii* to known amounts of these compounds.

The acid *E*, without the nucleotide, the tribasic acid, the mixture of hexa and hepta acids and the formaldehyde-treated cyanocobalamin were inactive or only slightly active for *Lb. leichmannii*. These compounds, or cobalt sulphate or cobalt mesoporphyrin, were added in 0·1 μg. amounts to 1 mg. SF 8 in distilled water. The mixtures were left at room temperature for 30 min. when 0·2 μg. cyanocobalamin was added. The volumes were made to 10 ml., the solutions ultrafiltered and the ultrafiltrates assayed for free cyanocobalamin. If the various compounds had combined with SF 8 the cyanocobalamin added subsequently would be free and able to pass into the ultrafiltrate. The amount of cyanocobalamin in these ultrafiltrates was compared with that present in the ultrafiltrate from a control experiment using SF 8 and cyanocobalamin. From this comparison it could be deduced whether the binding centres of SF 8 had previously been saturated by the compounds before the introduction of the cyanocobalamin.

Experiment using [^{14}CN]*cobalamin.* Cyanocobalamin in which the cyano group was labelled with ^{14}C, was a gift from Dr E. Lester Smith (Glaxo Laboratories Ltd.). Wet combustion of the material (specific activity approximately 0·2 μc/mg.) and gas counting of the CO_2 produced using a permanent vacuum line technique as described by Glascock (1954) showed that 50 μg. cyanocobalamin contained activity equivalent to $1{\cdot}77 \times 10^4$ counts/min.

A quantity of binding protein sufficient to combine with 1000 μg. cyanocobalamin was taken and mixed in solution with 500 μg. of the [^{14}CN]cobalamin. The mixture was immediately placed in a dialysis bag of cellophan tubing (closed at both ends) and immersed in distilled water. Four portions of 250 ml. water were used for dialysis over a period of 24 hr. To the combined dialysates were added 10 mg. of carrier KCN, the liquid was acidified to pH 3 with H_2SO_4, the HCN removed in a stream of N_2 and trapped in N-NaOH as in the method of estimating cyanide ion described by Boxer & Rickards (1951). The cyanide present in the NaOH was precipitated as AgCN, dried and counted by an end-window counter. After dialysis 500 μg. of unlabelled cyanocobalamin were added to fully saturate the binding factor and the cyanocobalamin–protein complex was purified as described in the preceding paper (Gregory & Holdsworth, 1955). A portion of the complex estimated to contain 50 μg. cyanocobalamin (25 μg. of the [^{14}CN]-cobalamin) by the spectroscopic method described earlier, was converted into CO_2 by wet combustion and the activity of the CO_2 produced was measured by gas counting.

RESULTS

The effect of specific group inhibitors on the combination of SF 8 with cyanocobalamin. The different reagents used to block specific groups on the protein molecule and the effect they had on the combination of SF 8 with cyanocobalamin are shown in Table 2. Of the reagents tested, fluorodinitrobenzene and iodine, at the higher concentrations, completely prevented the protein from combining with the vitamin. The query opposite formaldehyde treatment at pH 11 indicates that no cyanocobalamin could be measured in the ultrafiltrate. It was later found that, under the same experimental conditions, this concentration of formaldehyde at pH 11 (but not at pH 8) completely inactivated cyanocobalamin for *Lb. leichmannii*. In a repeat experiment, the solution of SF 8 and formaldehyde at pH 11 was dialysed to remove the excess formaldehyde before the cyanocobalamin was added. This treatment had no effect on the binding activity of the protein.

Table 2. *Effect of specific group inhibitors on the combination of SF 8 with cyanocobalamin*

The assay organism was *Lb. leichmannii*.

Amount of inhibiting reagent used/mg. SF 8 in 0·5 ml. of water	Effect on the combination of 1 mg. SF 8 with cyanocobalamin
1 ml. of 0·01 M *p*-chloromercuribenzoic acid	None
1 ml. of 0·01 M fluorodinitrobenzene	None
1 ml. of 0·17 M fluorodinitrobenzene	Complete inactivation
1 ml. of 3% formaldehyde at pH 8	None
1 ml. of 3% formaldehyde at pH 11	? (see text)
2 ml. of 0·01 N iodine in 0·05 M KI solution at pH 5·7	None
1 ml. of 0·01 N iodine in 0·01 M KI solution at pH 8·5	25% inactivation
*1 ml. of 0·1 N iodine in 0·1 M KI solution at pH 8·5	55% inactivation
*0·1 ml. of 0·5 N iodine in 0·5 M KI solution at pH 8·5	Complete inactivation
2 ml. of 0·01 M iodoacetic acid	None
8·5 ml. of 0·002 M *o*-iodosobenzoic acid	None
8·5 ml. of 0·002 M phenylmercuric acetate	None

* Dialysed for 6 hr. against running water and distilled water before the addition of cyanocobalamin.

Acetylation of SF 8 at both pH 8 and 10 by the method of Baddiley *et al.* (1952) did not alter its capacity to combine with cyanocobalamin. That the acetylation procedure was effective is apparent from Fig. 2, which shows that when crystalline serum albumin was acetylated, two substances were produced, both having greater electrophoretic mobilities than the original albumin. Furthermore, when acetylated SF 8 was saturated with [^{60}Co]cyanocobalamin, autoradiographs after electrophoresis showed that the cyanocobalamin was bound, and the resulting complex had a greater mobility than that formed from the untreated protein.

Combination of the altered cyanocobalamin molecule with SF 8. The ultrafiltration method of measuring binding activity showed that the monobasic acid *E*, the monobasic acid without the nucleotide, the tribasic acid, thiocyanatocobalamin and formaldehyde-treated cyanocobalamin all combined equally with SF 8. The only compounds tested that did not combine with the protein were the hexa- and hepta-acid decomposition products of cyanocobalamin and cobalt sulphate and cobalt mesoporphyrin.

Experiments with [^{14}CN]*cobalamin.* When cyanocobalamin, labelled with ^{14}C in the cyano group, was added to SF 8 and the mixture dialysed, no radioactivity appeared in the dialysate, indicating that the cyano group was not released during combination between vitamin and protein. Even after the considerable chemical manipulations involved in the isolation of the [^{14}CN]cobalamin–protein complex, the radioactivity in a portion of the material containing 50 μg. cyanocobalamin (25 μg. of the labelled vitamin) was $8{\cdot}55 \times 10^3$ counts/min., i.e. 96 % of the radioactivity of a corresponding amount of the original cyanocobalamin.

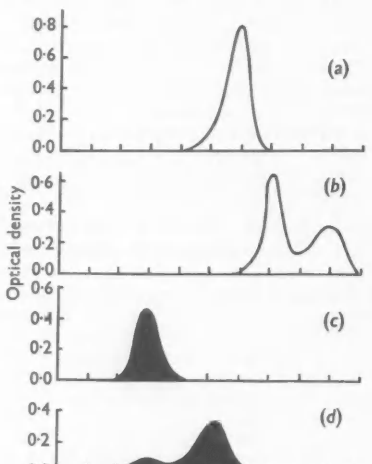

Fig. 2. Electrophoresis in sodium veronal buffer at pH 8·6: (*a*) pattern given by crystalline bovine serum albumin protein; (*b*) pattern given by acetylated bovine serum albumin protein; (*c*) autoradiograph of SF 8 with added [^{60}Co]cyanocobalamin; (*d*) autoradiograph of acetylated SF 8 with added [^{60}Co]cyanocobalamin.

DISCUSSION

The chemical examination of the cyanocobalamin–protein complex showed that the complex contained 16·1 % nitrogen and had an amino acid composition typical of proteins, except for a rather high tyrosine content. Since the anthrone method of Morris (1948) for carbohydrates does not estimate amino sugars, the complex contains 7 % carbohydrate in addition to the 9 % hexosamine. The presence of these sugar residues suggests that the binding substance is a glycoprotein.

The absorption curve clearly indicates the presence of both cyanocobalamin and a protein. The cyanocobalamin content, calculated from the height of the peak at 362 mμ. was 23·6 μg./mg. of complex. The quantity of material isolated was insufficient for application of any of the available methods for determining molecular weights. If it can be assumed that 1 molecule of cyanocobalamin combines with 1 molecule of protein, then the molecular weight can be calculated from the ratio of cyanocobalamin to protein in the complex. A molecular weight of 55 000 can be deduced for the protein from the presence of 23·6 μg. cyanocobalamin (mol.wt. 1300) in 1 mg. of the complex.

When this paper was being prepared for publication, a preliminary note by Wijmenga, Thompson, Stern & O'Connell (1954) reported the isolation of a similar cyanocobalamin–protein complex from pig gastric mucosa. The absorption curve we obtained for our complex was identical with that reported by Wijmenga *et al.* (1954), and yet they only found 12·3 μg. cyanocobalamin/mg. of the complex. From the sedimentation constant they concluded that the molecular weight of their complex was about 100 000.

For a quantitative microbiological estimation of the cyanocobalamin in our complex, we found that a preliminary digestion with papain or trypsin was necessary. Either of these two enzymes converts the microbiologically inactive complex into a form fully active for *Bact. coli* when heated with the basal assay medium, but having only 20 % of this activity when assayed unheated. Furthermore, ultrafiltration of the heated digest showed that not more than 10 % of the total cyanocobalamin had become

ultrafiltrable. Since cyanocobalamin itself can be ultrafiltered quantitatively, it appears possible that the digest contains peptide conjugates of cyanocobalamin.

Without the enzyme digestion, only 60% of the cyanocobalamin in the complex was available to *Bact. coli* after heat treatment. This fact may explain why Pitney, Beard & Van Loon (1954) were unable to recover, by heat treatment, all of the cyanocobalamin added to human serum.

In a previous paper we showed that the vitamin B_{12}-like compounds, factor *A*, factor *B* and pseudovitamin B_{12} were all bound by a protein concentrate from sow's whey (SF 8) and by an 'intrinsic factor' concentrate, to the same extent as cyanocobalamin (Gregory & Holdsworth, 1953). These vitamin B_{12}-like compounds differ from cyanocobalamin in their nucleotide groups. In factor *A*, the dimethylbenziminazole moiety of cyanocobalamin is replaced by 2-methyladenine (Brown & Smith, 1954) and in pseudovitamin B_{12} it is replaced by adenine (Pfiffner, Dion & Calkins, 1952). Removal of the nucleotide from cyanocobalamin, factor *A* or pseudovitamin B_{12} gives factor *B* (Gant, Smith & Parker, 1954). The nucleotide group, therefore, is not concerned in the combination of the vitamin with the protein.

The experiments reported in this paper have shown further that hydrolysis of the three primary amide groups in the cyanocobalamin molecule does not affect the ability of the vitamin to combine with the protein, but more drastic hydrolysis to the hexa and hepta acids (Armitage *et al.* 1953) gave a substance which no longer combined with the protein.

The experiments with [^{14}CN]cobalamin showed that the protein did not displace the cyano group when it combined with cyanocobalamin, and since thiocyanatocobalamin also combines with the protein, the linkage does not take place through the cyano group. The absorption spectrum of cyanocobalamin has a peak at 361 mμ. which shifts to 367 mμ. when the double cyanide complex is formed in the presence of excess cyanide ions. The absorption spectrum of the cyanocobalamin–protein complex has a peak at 362 mμ. which is unaffected by the addition of excess cyanide ions. This fact suggests that either the protein is linked to the vitamin in the place where the second cyano group normally adds on, or that steric hindrance by the protein prevents the cyanide ion from adding on to the cyanocobalamin in the complex. A more precise determination of the point of attachment of the protein to the vitamin molecule now awaits further information about the 'core' of the cyanocobalamin molecule.

The group on the protein molecule, concerned with its combination with cyanocobalamin, is not a sulphydryl group, since iodosobenzoic acid, iodoacetic acid, iodine acting as an oxidizing agent and *p*-chloromercuribenzoate had no effect on the binding activity of SF 8. Formaldehyde at pH 8 reacts with amino groups on proteins, and at pH 11 it attacks both amino and amido groups (Olcott & Fraenkel-Conrat, 1947). At neither pH did it affect the combination of SF 8 with cyanocobalamin. The reagent, 2′-acetylthioethylacetamide, is reported by Baddiley *et al.* (1952) to acetylate only amino groups on proteins, leaving hydroxyl groups unattacked. The acetylation of SF 8 with this reagent at both pH 8 and 10 had no effect on its combination with cyanocobalamin. However, these findings do not imply that an amino group on the protein is not concerned in its combination with the vitamin, since Baddiley *et al.* (1952) found that at pH 8 the reagent acetylated only one-quarter and at pH 10 one-half of the total free amino groups measured per mole of human serum albumin. If an amino group is concerned, it must be one that is not readily acetylated by this reagent or attacked by formaldehyde.

Fluorodinitrobenzene and iodine, at the higher concentrations used, were the only reagents that prevented the combination of SF 8 with the vitamin. Fluorodinitrobenzene, under the conditions used in these experiments, attacks amino and phenolic groups and to a lesser extent imidazole and sulphydryl groups (Olcott & Fraenkel-Conrat, 1947). Iodine acts as an oxidizing agent at an acid pH, in the presence of a high concentration of KI. With a low concentration and a higher pH, the oxidizing action of iodine is largely suppressed and iodination occurs. Under these conditions, iodination of the protein (presumably in the 3:5 positions of the tyrosyl residues) prevented its combination with the vitamin, and therefore it is possible that a phenolic group on the protein may be concerned in its linkage with cyanocobalamin.

SUMMARY

1. Chemical examination of the cyanocobalamin–protein complex isolated from sow's milk whey has shown that it contains 16·1% nitrogen, 7% carbohydrate and 9% hexosamine. Its amino acid composition is typical of proteins, except for a high tyrosine content. The absorption curve of the complex in aqueous solution has peaks at 278 mμ. (due to protein) and at 362, 410, 520 and 550 mμ. (due to cyanocobalamin).

2. The cyanocobalamin content of the complex is 23·6 μg./mg., calculated from the absorption curve, and 22·4 μg./mg. measured by *Escherichia coli*. Using these figures, and assuming that one molecule of protein combines with one molecule of cyanocobalamin a molecular weight of 55000 has been deduced for the protein.

3. The use of specific reagents to block different groups on the protein molecule, has shown that either a phenolic or an amino group on the protein may be concerned in its combination with the vitamin. The point at which the protein attaches itself to the cyanocobalamin molecule is also discussed.

We are indebted to Dr R. A. Kekwick of the Lister Institute for the gift of 2′-acetylthioethylacetamide and to Dr M. Dixon, F.R.S., of the School of Biochemistry, Cambridge, for the *p*-chloromercuribenzoate. In the experiments using [^{14}CN]cobalamin, the measurements of radioactivity were done by Dr R. F. Glascock, to whom we express our thanks. We also wish to thank Dr S. K. Kon for his interest in this work and valuable help in preparing it for publication. This paper forms part of a Ph.D. thesis presented by one of us (M. E. G.) to the University of Reading.

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An Apparatus for Continuous Electrophoresis on Paper

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The application of electrophoresis in solution with paper as a supporting medium, for separating and detecting substances which have different mobilities in an electric field is now well established. The technique to be described differs from others in that the mixture to be separated is continuously fed into the apparatus and the separated components can be continuously removed, i.e. it can be used as a preparative method. An attempt was made by Philpot (1940) to use electrophoresis in a thin horizontal layer of solution between layers of electrolyte, as a continuous process. Some of the difficulties of this technique were overcome by Svensson & Brattsten (1949), by using a thin vertical layer and powdered glass to prevent mixing during the separation. There was a constant flow of buffer solution down through the glass and out through a series of tubes at the bottom of a narrow Perspex box. If a constant supply of material was applied at one place on the top surface it was carried down by the buffer solution to the outlet immediately below. However, if a potential gradient was applied at the ends of the box the substance was deflected towards one electrode and appeared at some other outlet, depending on its rate of movement in the electrical field. With a mixture of substances of different mobilities a continuous separation could be effected. The apparatus seems to have found very little practical application, probably owing to the difficulty of setting up and also to the poor resolution obtainable. These workers also tried a sheet of filter paper as a supporting medium for the electrolyte but with little success. Grassmann & Hannig (1950) were the first to use paper successfully in this type of apparatus. Platinum wire electrodes were fixed to the sides of the paper and the buffer solution was allowed to drip off at a series of points. Successful continuous separations of amino acid mixtures and proteins were reported. A forerunner of this type of apparatus was that of Haugaard & Kroner (1948), who carried out paper chromatography on a spot of the material to be separated and applied an electric

field across the paper, at right angles to the flow of solvent. Separation was achieved by two separate processes, chromatography in the downward direction and electrophoresis horizontally, but the process in this technique was discontinuous. A slightly different type of apparatus was described by Durrum (1951), who obtained continuous separation of dyestuffs, amino acid mixtures and serum proteins. Strain & Sullivan (1951) have used this method for inorganic preparative work. An apparatus incorporating improvements but based on these previous models was demonstrated to the Biochemical Society (Holdsworth, 1953), and has been successfully used for the preparation of pure milk proteins (cf. Gregory & Holdsworth, 1955). A recent publication of Grassmann & Hannig (1953) has dealt comprehensively with the theory and practice of this technique. Although simple in principle the successful continuous operation of this method must overcome numerous technical difficulties, and the way in which many of these have been overcome in this laboratory is described in this paper.

PRINCIPLE

When the paper is acting solely as an inactive supporting medium, the angle to the vertical of the path taken by a particular component is α, where

$$\tan \alpha = \frac{\text{Rate of movement in the electrical field}}{\text{Rate of movement down the paper (i.e. of the buffer solution)}}.$$

It is evident that for this method to be successful it is essential to have close control over all the variables which will be considered in turn.

(i) The electrical field strength must be constant. The application of the field to only the bottom corners of the paper (Durrum, 1951) even when this has extra thicknesses at the edges (Holdsworth, 1953) means that the potential difference between the two sides steadily diminishes as the top of the paper is approached. For long periods of operation it is convenient to have a uniform field, therefore the electrodes must extend down the edges of the paper (cf. Grassmann & Hannig, 1953).

(ii) The rate of flow of the electrolyte down the paper and its composition must be kept constant. The supply of electrolyte is usually provided by allowing the paper to dip into a trough of electrolyte supplied with a constant-level device. On its passage down the paper the electrolyte is subjected to the heating effect of the current, which causes evaporation of the water. Thus non-volatile solutes, including buffer salts become more concentrated in their progress down the paper. For many purposes it is possible with a sufficiently rapid rate of flow to achieve a steady state; the only disadvantage of this concentrating effect then is that the path followed by a substance in the electrical field is non-linear. By cooling the paper (as in the apparatus to be described) this effect is eliminated and heat-labile materials are not destroyed.

(iii) The products of electrolysis must not be allowed to bring about a change of pH on the paper. The removal of these products by arranging an extra flow of buffer down the edges of the paper (Holdsworth, 1953; Grassmann & Hannig, 1953) is not entirely satisfactory and in the apparatus described below the electrodes are continually washed with fresh buffer solution and are separated from the paper by a cellophan membrane.

(iv) The methods described so far have made no attempt to control the temperature of the system, reliance has been on the achievement of a steady state when the external temperature was kept constant. Even with low current densities the use of the high voltages necessary for separation results in significant increases of temperature in the apparatus, and therefore it was deemed advisable to remove the heat produced on the paper, in addition to having a constant external temperature.

DESCRIPTION OF THE APPARATUS

A general view of the apparatus is shown in the photograph (Fig. 1), and Figs. 2 and 3 show the constructional details. The apparatus is made of $\frac{1}{4}$ in. Perspex sheet and consists essentially of a framework (*A*) which, when the removable back, top and two front panels (*B*) are in position, forms the moisture-saturated enclosure for the paper. Any moisture collecting on the panels is conducted away by the gutters (*D*).

The cooling system (*T*) is a Perspex box with the front face $\frac{1}{16}$ in. thick. The whole of this face is cooled by water entering at *V*, passing round a series of baffles and eventually leaving at *W*. The Perspex cooler is attached by four stainless steel bolts to the central strut of the framework *C*, but separated from the strut by spacing units (*U*) of Perspex. The two spacers at the bottom are $\frac{3}{4}$ in. thick; those at the top $\frac{9}{16}$ in. thick. This slight tilt of the cooler from the vertical ensures that the paper clings to the entire surface.

The trough *E* measures $18 \times 2 \times 1\frac{1}{2}$ in. high (internal) and along the top of one face is cemented a $\frac{1}{4}$ in. diameter Perspex rod (*F*) over which the paper hangs. A constant head of electrolyte is ensured by a continual drip from the feed (*P*) into the trough, the excess passing out of the overflow (*G*) into a tank (*H*) of capacity about 2 l. A pump returns this buffer to the supply vessel, which is a 5 l. aspirator bottle. The solution drips off the paper at 29 points into small glass funnels (*K*) constructed from Pyrex tubing of internal diameters 9 and 3 mm. The centres of these funnels are $\frac{1}{2}$ in. apart and are held in a Perspex rack (*L*). Fractions are collected either in 150×9 mm. test tubes or into larger vessels. By removing the lower front panel a rack of test tubes (*N*) may be replaced by fresh tubes daily, without disturbing the paper, since the lower part of the funnels (*K*) consists of lengths of flexible polyvinyl chloride (P.V.C.) tubing. The first and last drip points (see Fig. 4) serve a wide strip of paper, and the liquid is collected in correspondingly larger vessels.

Support for the electrodes is provided by strips of Perspex 17 × 1 × ¼ in. which have a U-shaped channel milled out ⅛ in. deep, ⅜ in. across and ⅛ in. from one edge (*I*). The electrodes are 20 in. lengths of 0·38 mm. platinum wire (*S*) enclosed in a length (approx. 16 in.) of cellophan tubing (*Q*) (⅝ in., Visking Corporation). The bottom of the cellophan tubing is tightly bound with linen thread over a 2 in. length of 6 mm. diameter glass tube covered with flexible P.V.C. tubing and is connected to the outlet of a Perspex reservoir (*J*) 3 × 2½ × 3 in., placed on the same shelf which supports the trough *E*. The top end of the cellophan tube is fastened, in the same way as the lower end to the glass connexion (*R*) (Fig. 3) made from 6 mm. Pyrex tubing. This construction is necessary to allow for the introduction of the platinum electrodes (*S*), for the escape of gases produced by electrolysis and for the overflow of electrolyte. The cellophan tubing is fastened to the Perspex supports (*I*) where they are stiffened by the underlying glass connecting tubes by clips made from ⅛ in. diameter stainless-steel rod covered with P.V.C. tube. A diagram of this flowing electrode system is shown in Fig. 3. The flattened, circular centre portion of *R* is displaced slightly backwards from the plane of the cellophan tubing and the paper, so as to afford no obstruction to the 'hang' of the paper from the trough. The overflow from anode and cathode is taken to the Perspex tank *H*, where it mixes and is returned to the aspirator bottle by the pump.

It is necessary to discharge the two overflow tubes into the top of the tank *H* well above the level of the electrolyte to eliminate the possibility of a short circuit. To ensure a good contact between the paper and the cellophan tubing, strips of Perspex 15 × ⅜ × ¼ in. press the paper against the electrodes and are kept in place by stainless steel clips. Electrolyte is added dropwise, to prevent short circuiting, to the reservoir vessels *J*. In this way a continuous change of liquid surrounding the platinum wire is obtained. Furthermore, the paper is separated from the electrodes by a cellophan membrane, which ensures that pH changes do not affect the paper.

The paper is cut from a piece of 18¼ × 22½ in. filter paper to the dimensions shown in the diagram (Fig. 4). The 3 in. portion at the top is doubled back to dip into the trough and is kept in position by a thick glass rod. The surface of the cooler (*T*) is treated with Silicone M.S. 550 to make it water-repellent. The electrolyte is sprayed on the paper, which is pressed against the surface of the cooler eliminating all air pockets. The paper points are then pushed into the glass funnels making sure that the points adhere to the glass by capillary attraction, since only with a good contact can there be an even flow of electrolyte from the paper. With trough *E* full of electrolyte and the apparatus closed, steady state conditions of flow down the paper and saturation of the atmosphere are reached in about 4 hr.

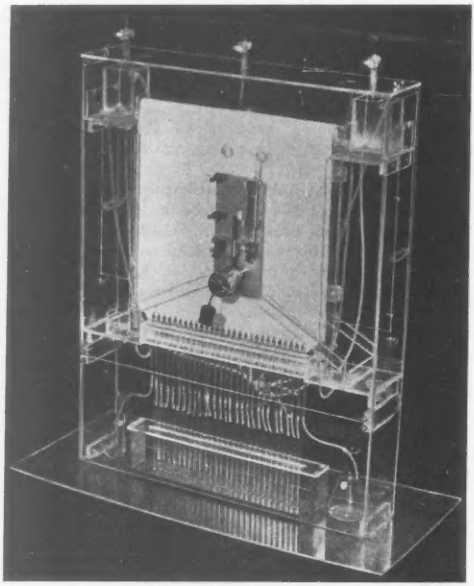

Fig. 1

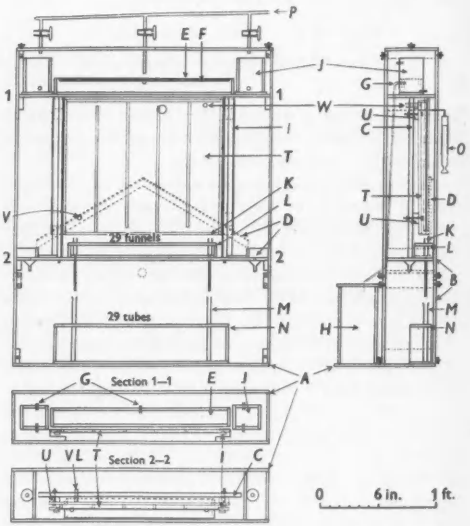

Fig. 2

Fig. 1. Apparatus for continuous electrophoresis on paper.

Fig. 2. Diagram of the apparatus for continuous electrophoresis on paper. *A*, frame; *B*, front panels; *C*, strut; *D*, condensate collector; *E*, trough; *F*, rod; *G*, overflow; *H*, tank; *I*, electrode supports; *J*, reservoirs; *K*, funnels; *L*, funnel holder; *M*, tubes; *N*, tube holder; *O*, syringe; *P*, electrolyte feed; *T*, cooling system; *U*, spacing pieces; *V*, water inlet; *W*, water outlet.

Temperature control. The apparatus is used in a room where the temperature is controlled at 20±1°. The cooling system for the paper is supplied from a large glass tank of water 20 × 20 × 10 in. cooled by a glass coil fed from the cold tap. The temperature of this reservoir is then maintained at 19° (i.e. 1° less than ambient temperature) by a Circotherm pump and heater unit (Shandon Scientific Co.).

Method of applying the test solution. The usual method of applying material to be separated by a wick of filter paper 1 mm. wide (Durrum, 1951; Grassmann & Hannig, 1953) is not very suitable for long periods of operation and a mechanically operated syringe was made in the Engineering Department, of this Institute, by Mr E. J. Pinnegar. Experience has shown that the system can deal with material at the rate of 1–5 ml. per 24 hr. By using either a 20 ml. or a 10 ml. Chance's interchangeable all-glass syringe, amounts of 4 or 2 ml. can be delivered in 24 hr. by the arrangement, shown in Fig. 1.

Two glass syringes are selected, ejecting 2 and 4 ml. respectively for a plunger travel of 0·480 in., the barrels of which are attached by four 'Terry' spring clips to a brass base-plate 9 × 4 × 18 in. s.w.g. Spring clips are also used to attach the plunger handles to the ends and at right angles to a carrier bar $3\frac{3}{4} \times \frac{5}{16} \times \frac{1}{8}$ in., the centre of which is silver-soldered at 90° to a $\frac{5}{16}$ in. inside diameter brass carrier tube $1\frac{1}{2}$ in. long. Into this a $\frac{5}{16}$ in. outside diameter, 10 square threads/in. worm, $3\frac{1}{2}$ in. long, is fitted. A spring-loaded pawl is fixed to the carrier tube to mesh with the worm and provides a linear drive to the syringe plunger. The worm is driven by a 2 rev./hr. Sangamo Weston motor unit through a worm-and-pinion gear, providing a speed reduction of 10:1. The reduction gear pinion is assembled on the carrier worm spindle, which is then mounted between two bearing brackets bent up from the base-plate, and the motor unit with the reduction worm pinned to the motor spindle is then screwed to the base-plate with reduction worm and pinion in mesh.

To fill the syringes it is necessary only to depress the spring-loaded pawl and draw the brass carrier tube down to the stops provided.

The chosen syringe is fitted with a glass right-angled needle, but to prevent the paper being pierced, the final contact between needle and paper is by a short length of flexible P.V.C. tubing, 0·5 mm. bore. The brass plate is fastened to the front Perspex panel (Fig. 1) by two stainless steel 8 B.A. bolts, and a hole is bored through the panel for entry of the needle. Two positions, one central and the other one-third of the way from one edge of the paper are suitable for most requirements, particularly since the polarity of the electrodes can be reversed. The point of application of the material is 2 in. below the lip of the trough *E*.

Power supply. A source of d.c. voltage stabilized by three STV 780/40 glow discharge tubes in series (Benson, 1950) was used to provide voltages up to 840 v in steps of 70 v. Change of load in the range 2–30 mA causes a change of less than 1% in the potential across the paper.

OPERATION OF THE APPARATUS

The point at which a component will drip off the paper is determined by the electrophoretic mobility, the composition of the electrolyte, the voltage across the paper and the rate of movement down the paper, which in turn depends on the grade of paper used.

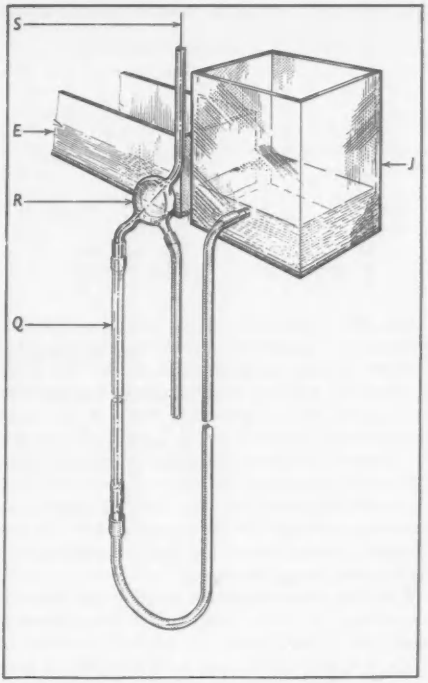

Fig. 3. Diagram of the flowing electrode system. *E*, *J*, as Fig. 2; *Q*, cellophan bag; *R*, flattened circular section; *S*, Pt wire.

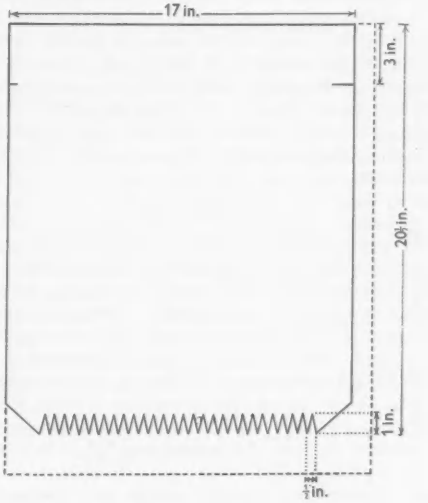

Fig. 4. Diagram showing dimensions of the paper for continuous electrophoresis.

Table 1. *Some Whatman papers suitable for continuous electrophoresis*

Paper	Characteristics	Remarks
No. 1	Thin, slow	For small quantities of substances of low mobility, e.g. some carbohydrates in borate buffers
No. 2	Medium-thick, slow	As above, but capable of dealing with larger amounts of material
No. 54	Thin, medium speed	A tough paper very useful for small amounts of protein, e.g. enzymes, also for coenzymes or peptides
No. 3 MM	Thick, medium speed	For the majority of protein separations
No. 4	Thin, fast	Can be used for proteins, peptides and sugars
No. 31	Thin, very fast	For substances of high mobility, e.g. inorganic ions, organic acids and bases, dyestuffs, etc.
No. 31 extra thick	Thick, very fast	

A high flow rate will enable larger amounts of material to be processed. However, the separation between various components increases the longer the material remains on the paper, and the final choice must be a compromise. Some of the papers used successfully are shown in Table 1. The electrolyte chosen depends on the pH at which separation of the components can be achieved. Since with the apparatus described here the heating effect of the current is dissipated by the cooling systen, the ionic strength does not have to be kept very low as with the free-hanging paper strips.

With the chosen paper in position and wet with electrolyte, the drip feed to the three reservoir vessels is adjusted and the current switched on. When a steady state has been established (after 4 hr.) the mechanically operated syringe is charged with a solution of the substance to be separated (dissolved in the electrolyte), fixed into position and the synchronous motor started. Where no adsorption takes place quantitative separations can be obtained by collecting fractions for several hours after all the contents of the syringe have been placed on the paper. However, it is often convenient to stop the process before the material has been completely washed off the paper, which is dried and stained to serve as a guide to the required fractions.

CONCLUSIONS

The technique of one-dimensional electrophoresis on paper has been employed for the detection of small quantities of a wide range of compounds, but is inconvenient for the preparation of larger amounts of material. The apparatus described in this article has been used for the continuous separation of mixtures in amounts up to 4 ml. of a 5 % solution/24 hr. When the supporting medium is a thin sheet of close-textured paper, sharp resolution can be obtained. A little of this sharpness is lost by addition of the cooling system, but as this prevents accumulation of electrolyte owing to loss of water by evaporation, the position at which a substance drips off the paper is more stable over long periods of operation. When using paper from one batch, substances appear in the same tubes in experiments conducted at different times. The use of paper as a support has certain disadvantages. In protein separations, some proteins become strongly adsorbed on paper at pH values below 7, thus limiting the range within which the process can be used. This difficulty could possibly be overcome by pretreatment of the paper, e.g. by esterification of carboxylic groups. There is no apparent reason why fabric made of fibres other than cellulose should not prove useful. Geldmacher-Mallinckrodt & Weinland (1953) have already demonstrated that one-dimensional separations of polysaccharides can be achieved by electrophoresis on closely woven silk.

The apparatus should be particularly useful for the further purification of materials when established procedures are cumbersome or inefficient. For instance, in the isolation of a cyanocobalamin–protein from milk a stage was reached when ammonium sulphate fractionation gave no further purification and the proteins contained only 1 % of the required material. This mixture was readily resolved by continuous electrophoresis to give cyanocobalamin–protein free from the majority of the other proteins (see Fig. 4, Gregory & Holdsworth, 1955).

SUMMARY

Details are given of the construction of an apparatus for continuous electrophoresis on paper. By means of a flowing electrode system long periods of operation can be used without pH change on the paper. Arrangements for cooling the paper allow the technique to be applied to heat labile materials (e.g. enzymes) and also lead to more reproducible results, since evaporation of solvent from the electrolyte is eliminated.

The polyvinyl chloride tubing was obtained from A. R. Horwell, 18 Christchurch Avenue, London, N.W. 6, Silicone fluid MS 550 from Hopkin and Williams, Chadwell Heath, Essex, the Sangamo motor unit, model 57, 2 rev./hr., was obtained from Sangamo-Weston, Enfield, Middlesex. The pump for returning electrolyte to the aspirator bottle was

a Stuart-Turner pump in which the parts coming in contact with electrolyte were replaced by similar parts made of Perspex.

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Antithyroid Substances

3. DERIVATIVES OF 2-MERCAPTOGLYOXALINES

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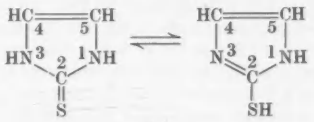
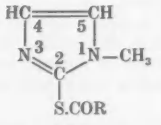
In Parts 1 and 2 (Searle, Lawson & Hemmings, 1950; Searle, Lawson & Morley, 1951) the examination of a range of 2-mercaptoglyoxalines (I), 2-mercaptothiazoles and 2-thiohydantoins for antithyroid activity in young rats was described.



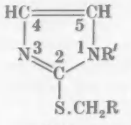
(I)

As a result of chemical investigations in the 2-mercaptoglyoxaline series a number of new compounds have been obtained, and these have now been subjected to screening tests for antithyroid activity by the methods already described, namely by determining the effect of a single dose of the drug on the radioactive iodine uptake of the rat's thyroid gland over a 4 hr. period.

With a view to increasing the duration of activity of some antithyroid drugs, particularly the highly active 2-mercapto-1-methylglyoxaline ('methimazole', 'mercazole'), the effect of introducing a hydrolysable grouping on the sulphur atom has been investigated. Results obtained with one such compound, 2-ethoxycarbonylthio-1-methylglyoxaline ('carbimazole', 'neomercazole') (II, R = OC_2H_5) were described by Lawson, Rimington & Searle (1951). This substance is at least as active in the rat as its parent thiol, but no evidence was obtained that it was hydrolysed sufficiently slowly *in vivo* to give a markedly increased duration of activity. In clinical use (Doniach, 1953; Poate, 1953), however, it appears to be slightly more active than 2-mercapto-1-methylglyoxaline, and the substance is free of the bitter taste associated with the thiol drugs.



(II)



(III)

A preliminary account has appeared of the antithyroid activity of some further S-substituted derivatives of 2-mercaptoglyoxalines (Lawson & Searle, 1954). Reaction of 2-mercapto-1-methylglyoxaline with methyl chloroformate, benzyl chloroformate or phosgene yields derivatives (II) which are readily hydrolysed *in vitro* back to the thiol, and which show high antithyroid activity in the rat. Reaction of mercaptoglyoxalines with alkyl halides, on the other hand, yields thioethers (III) which can only be hydrolysed back to the parent thiols under much more drastic conditions.

It is usually assumed that antithyroid agents such as thiourea and the thiouracils and mercaptoglyoxalines owe their activity to the presence of the potentially free thiol group, which by its ready reduction of elementary iodine to iodide ion

$$2R\text{–}S^- + I_2 \rightarrow R\text{–}S\text{–}S\text{–}R + 2I^-$$

interferes with the iodination of tyrosine in the thyroid protein and hence inhibits the synthesis of the thyroid hormone.

2-Alkylthioglyoxalines would therefore be expected to be devoid of antithyroid activity. It was observed by Searle *et al.* (1950), however, that 2-benzylthioglyoxaline showed slight activity, and some 2-methylthio- and 2-benzylthio-glyoxalines have therefore been examined in the present work. A number of them have been found to show low but definite activity.

Ergotamine has been shown to lower the heat-production of the thyroid-fed dog (Bouckaert, 1926) and of the normal rabbit (Marine, Deutch & Cipra, 1927), and has been used in the treatment of hyperthyroidism. It was therefore thought of interest to examine the effect of this drug and of dihydroergotamine on thyroidal iodine uptake by the radioactive iodine technique.

METHODS

The assay technique was similar to that already described (Searle *et al.* 1950).

Rats. Newly weaned hooded rats of Glaxo stock were maintained for some weeks before use on Glaxo powder diet RBSS-9, the low I_2-content of which results in thyroidal ^{131}I-uptakes of the order of 15–25% over 4 hr. in the control animals. The rats were used in groups of about seven animals, matched for weight and sex. Occasionally batches of these rats did not grow well on the diet, and in some experiments, therefore, rats bred at the Royal Free Hospital School of Medicine have been used.

Drugs. The synthesis of new compounds will be described elsewhere. In the preparation of *S*-methyl derivatives of mercaptoglyoxalines the use of methyl iodide was avoided as any I_2 contamination would cause misleading results.

2-Mercaptohistamine (4-2′-aminoethyl-2-mercaptoglyoxaline) was prepared from butyne-1:4-diol as described by Fraser & Raphael (1952). Attempts to obtain the 1-methyl analogue by condensing the 1:4-diaminobutan-2-one with CH_3NCS instead of KCNS were unsuccessful.

Compounds for testing were administered by stomach tube, usually at a level of 0·05 m-mole/kg. body weight in the case of free thiols and *S*-acyl derivatives but at higher levels with *S*-alkyl derivatives. They were given in aqueous solution, as the Na or HCl salts when not otherwise soluble. Ergotamine and dihydroergotamine (Sandoz Products Ltd.) were injected subcutaneously as the tartrate ('Femergin') and methanesulphonate respectively in 0·9% NaCl solution. The control rats were dosed similarly with water or saline solution.

Radioactive iodine. One hour after dosing the drug under test, the rats were injected intraperitoneally with 0·5 ml. 0·9% NaCl, containing *ca.* 2 μc of ^{131}I per ml. In some experiments this time interval was increased to 20 hr.

After a further 4 hr. the animals were killed with $CHCl_3$. The thyroids were dissected out, weighed, allowed to stand overnight in 2 ml. 2% NaOH, and dissolved by boiling gently. The solutions were diluted to 10 ml. with water, and the radioactivity in each rat's thyroids was then determined using an M 6 Geiger–Müller liquid counter (20th Century Electronics).

Results obtained are summarized in Table 1.

RESULTS AND DISCUSSION

2-*Mercaptoglyoxalines*

In previous experiments (Searle *et al.* 1950, 1951) it was found that all substituents in the 4- and 5-positions of 2-mercaptoglyoxaline caused reduced antithyroid activity, the loss of activity being least in the presence of a single 4-alkyl-substituent and almost complete with a strongly polar group, such as free or substituted amino or hydroxyl. The results obtained in the present experiments are in accord with this previous work.

The complete series of 4-alkyl-substituted derivatives from 2-mercaptoglyoxaline to 4-*n*-hexyl-2-mercaptoglyoxaline has now been tested by the same method. By assuming all the compounds to have dose/response curves similar to that of 2-mercaptoglyoxaline (Searle *et al.* 1950), the dose of each which would cause a 50% depression in radioactive iodine uptake can be estimated. Taking the activity of 2-mercaptoglyoxaline as unity, the relative activities of the 4-alkyl derivatives are found to be: methyl, 0·2; ethyl, 0·5; *n*-propyl, 0·5; *n*-butyl, 0·5; *n*-pentyl, 0·2 and *n*-hexyl, 0·15.

Activity thus falls fairly steadily with increasing chain-length, the only anomaly being the 4-methyl derivative. The abnormally low result with this compound might be due to partial oxidation to the inactive 4-carboxylic acid, or to the hyperconjugation effect causing increased electron release into the ring, which seems to be a factor causing reduced antithyroid activity. The introduction of an *iso*-propyl or a 1-formylethyl group into the 1-position of the 4-methyl compound reduced its activity slightly.

The antithyroid activities of some 4-alkyl-substituted mercaptoglyoxalines were estimated using a histological method by Miller & Bergmann (quoted by Jackman, Klenk, Fishburn, Tullar & Archer, 1948). They also found a reduced activity relative to 2-mercaptoglyoxaline in the *iso*propyl, *iso*butyl, *n*-pentyl, *n*-hexyl, *cyclo*pentyl, *cyclo*hexylmethyl and benzyl derivatives, but they found the *n*-propyl compound to be approximately 3 times as active as its parent.

The increased uptake (+48·5%) found in an early experiment with the diethoxycarbonyl derivative of 4-aminomethyl-2-mercaptoglyoxaline (Searle *et al.* 1951) could not be repeated in the present work.

Substances with amino or hydroxyl groups in the 4- and 5- positions were inactive as expected from previous experiments. It had been thought possible that an aldehydic group in these positions would enhance activity, but this was found not to be the case with 4-formyl-2-mercaptoglyoxaline, possibly owing to oxidation to the inactive carboxylic acid.

Table 1. *Effect of various compounds on* ^{131}I*-uptake of the rat's thyroid gland over a period of 4 hr.*

Compound	Dose (mg./kg.)	No. of rats dosed	Depression of ^{131}I-uptake (%)	Significance of difference from controls*
2-Mercaptoglyoxalines				
2-Mercaptoglyoxaline	1	5	53·8	A
	†(5	6	2·2)	C
4-*n*-Butyl-2-mercaptoglyoxaline	0·2	6	−53·8	A
	1	5	35·0	B
	5	6	87·7	A
	8	6	94·2	A
	(8	6	13·3)	C
4-*n*-Hexyl-2-mercaptoglyoxaline	9	8	62·4	A
	(9	6	1·8)	C
4-Benzyl-5-ethyl-2-mercaptoglyoxaline	11	7	9·9	C
4-Ethyl-5-*p*-hydroxybenzyl-2-mercaptoglyoxaline	12	5	27·8	B
2-2′-Mercaptoglyoxalin-4′-yl propionic acid	8·5	5	19·5	C
2-Mercapto-4-2′-methylthioethylglyoxaline	8·5	8	18·9	C
4-*ω*-Amino-*n*-butyl-2-mercaptoglyoxaline	10·5	8	20·0	C
4-4′-Glyoxalinylmethyl-2-mercaptoglyoxaline	9	7	10·0	C
4-Formyl-2-mercaptoglyoxaline	6·5	7	−4·15	C
	50	6	30·5	B
2-Mercaptohistamine	9	6	22·3	C
4-2′-Aminoethyl-2-mercaptoglyoxalin-5-yl acetic acid	10	5	−20·8	C
1-1′-Formylethyl-2-mercapto-4-or 5-methylglyoxaline	8·5	6	43·8	B
	(8·5	5	5·6)	C
4(5)-Ethoxycarbonylaminomethyl-2-ethoxycarbonylthioglyoxaline	14	6	19·3	B
	30	7	60·5	A
2-Mercapto-4-methyl-1-*iso*propylglyoxaline	8	8	48·1	A
2-Mercaptoglyoxaline–maleic anhydride adduct	11	8	12·6	C
4- or 5-*n*-Butyl-1-1′-formyl-*n*-pentyl-2-mercaptoglyoxaline	13	6	21·1	C
S-Acyl 2-mercaptoglyoxalines				
2-Methoxycarbonylthio-1-methylglyoxaline	0·2	7	57·5	A
	1·0	5	89·0	A
2-Benzyloxycarbonylthio-1-methylglyoxaline	1	5	86·2	A
	(12	7	23·8)	C
2-Benzamidomethylcarbonylthio-1-methylglyoxaline	14	6	97·3	A
Di(1-methylglyoxalin-2-yl)dithiolcarbonate, 2HCl	8	5	96·8	A
	14	6	94·7	A
2-Acetylthio-4(5)-methylglyoxaline	11	7	93·0	A
	11	6	71·0	A
S-Alkyl 2-mercaptoglyoxalines				
2-Benzylthioglyoxaline	50	6	38·5	B
	100	6	55·9	A
2-Methylthioglyoxaline	50	6	52·5	B
2-Benzylthio-1-methylglyoxaline, HCl	25	7	41·8	A
	50	6	52·7	B
	(12	8	11·2)	C
1-Methyl-2-methylthioglyoxaline, HCl	25	7	23·6	B
	50	6	26·0	C
	(8	7	−4·1)	C
2-Ethoxycarbonylmethylthio-1-methylglyoxaline	10	6	13·8	C
2-Benzylthio-4-methylglyoxaline	50	6	51·8	A
	100	7	74·6	A
4-Methyl-2-methylthioglyoxaline	50	8	48·5	A
4′-Methylthiazolo-(2′:3′:2:1)5-methylglyoxaline	7·5	6	13·9	C
	(7·5	5	2·6)	C
Miscellaneous				
Thiourea	15	7	87·8	A
S-Benzylthiourea, HCl	10	6	27·0	B
	50	7	51·6	B
3:5-Diaminothia-2:4-diazole	6	6	−6·3	C
Ergotamine tartrate	0·5	7	19·7	C
	0·5	12	41·7	B
	1·0	7	24·8	B
	1·0	16	43·8	A
	1·0	18	34·6	A
Dihydroergotamine methanesulphonate	1·0	11	−1·7	C

* The significance of the uptake depression was examined by Student's *t* test, and is expressed as follows; $P < 0{\cdot}001$, A; $0{\cdot}1 > P > 0{\cdot}001$, B; $P > 0{\cdot}1$, C.

† Parentheses denote those experiments in which ^{131}I was administered 20 hr. after the drug under test. In all other cases the interval was 1 hr.

It would be interesting to examine a 4-nitro derivative in this connexion, but it has not been found possible to prepare such a compound.

Duration of activity

On increasing the length of the carbon chain in the 4-alkyl-substituted 2-mercaptoglyoxalines, the compounds become increasingly soluble in organic solvents and insoluble in aqueous media. It was thought possible that by virtue of being fat-soluble some higher 4-alkyl-2-mercaptoglyoxalines might have a more prolonged action than the drugs in common use. Moreover, on a molecular basis 4-*n*-butyl-2-mercaptoglyoxaline was the most active 4-substituted mercaptoglyoxaline in the usual radioactive iodine uptake test.

The most satisfactory way of investigating the duration of activity of antithyroid substances would be by a neck-counter method as developed for tests on human subjects by Astwood and co-workers (see, for example, Stanley & Astwood, 1947). Such a method could not be employed in the present work, but it was thought of interest to find the effect of increasing the time interval between administration of the drug and the radioactive iodine from 1 to 20 hr. It was found, however, that at levels of 0·05 m-mole/kg. body weight, 2-mercaptoglyoxaline and also its 4-*n*-butyl and 4-*n*-hexyl derivatives were quite without effect on the uptake of ^{131}I injected after 20 hr.

S-Acyl-2-mercaptoglyoxalines

On allowing for its greater molecular weight, 2-ethoxycarbonylthio-1-methylglyoxaline was found to have antithyroid activity very similar to that of the parent thiol when tested in the rat by the technique already described at a number of dosage levels (Lawson *et al.* 1951). When the interval between dosing the drug and injecting radioactive iodine was increased from 1 to 20 hr. (see preceding section) the 4 hr. uptake depressions were similarly reduced, and it thus seems that the ethoxycarbonyl derivative is hydrolysed to the thiol too quickly in the rat's body for the duration of antithyroid activity to be markedly prolonged.

High activity, similar to that of the parent 2-mercapto-1-methylglyoxaline, has similarly been found in the following compounds (where R denotes the 2-substituted 1-methylglyoxalinyl radical);

$R.S.CO.OCH_3$, $R.S.CO.O.CH_2.C_6H_5$,

$R.S.CO.CH_2NH.CO.C_6H_5$

and $R.S.CO.S.R,2HCl$.

Like the *S*-ethoxycarbonyl derivative, the *S*-benzyloxycarbonyl compound showed no evidence of any prolonged activity in the test with the 20 hr. time interval.

S-Alkyl-2-mercaptoglyoxalines

2-Benzylthioglyoxaline has been examined at higher doses (50 and 100 mg./kg.) than that reported earlier (Searle *et al.* 1950). The *S*-methyl derivative has been examined similarly, as have the *S*-benzyl and *S*-methyl derivatives of 2-mercapto-1-methyl- and 2-mercapto-4-methyl-glyoxaline. At these levels marked depressions of radioactive iodine uptake are obtained, the highest being 75% following dosage of 2-benzylthio-4-methylglyoxaline at 100 mg./kg.

By utilizing the previously reported results for the free mercaptoglyoxalines at various dose levels it is possible to calculate approximately for each experiment with an *S*-alkyl mercaptoglyoxaline the dose of the corresponding thiol which would give a similar depression in radioactive iodine uptake (Table 2).

The interesting fact emerges that the substitution of a methyl or benzyl grouping on the sulphur atom may produce much less reduction in antithyroid activity in this test than expected. In particular, 2-benzylthio-4-methylglyoxaline has about one-tenth of the activity of the corresponding thiol, and *S*-benzylthiourea hydrochloride one-third to one-eighth of the activity of thiourea. The more active drugs 2-mercaptoglyoxaline and 2-mercapto-1-methylglyoxaline showed a much greater reduction in activity on blocking the sulphur atom, but this seems mainly due to the greater activity of the thiols as the activities of the thioethers are of the same order of magnitude.

The activity of the 2-alkylthioglyoxalines is probably a property of the substances as such and not due to their relationship to the highly active mercaptoglyoxalines. The activity could be due to the reaction of the compounds with iodine in the thyroid gland so that there is a reduced amount of iodine available for thyroid hormone synthesis. Any substance which can reach the thyroid and which can be iodinated can theoretically act in this way, and in the case of resorcinol this mechanism has been demonstrated by the chromatographic identification of iodoresorcinol after incubation of resorcinol with rat-thyroid slices (Fawcett & Kirkwood, 1953). Though in the present work 2-benzylthio-4-methylglyoxaline has been iodinated giving the 5-iodo derivative (m.p. 153–154°, decomp.), reaction of these substances with iodine is complex and pure products are difficult to isolate.

The alternative possibility that the *S*-alkyl derivatives can be converted into thiols in the body was thought unlikely, since *in vitro* this change can only be brought about by boiling with concentrated hydrochloric acid. Moreover, there are no recorded instances of this type of debenzylation *in vivo* (Prof. R. T. Williams, private communication). Fission of the glyoxaline–sulphur link occurs more easily

Table 2. *Antithyroid activity of mercaptoglyoxalines and thiourea compared with that of their S-benzyl and S-methyl derivatives*

Compound	Depression of ^{131}I uptake (%)	Dose (mg./kg.)	Approx. dose of thiol to give same depression (mg./kg.)	Ratio: Dose thioether / Dose thiol
2-Mercaptoglyoxaline				
S-Benzyl	39	50	1	50
	56	100	1·5	33
S-Methyl	53	50	1·5	33
2-Mercapto-1-methylglyoxaline				
S-Benzyl hydrochloride	42	25	0·15	170
	53	50	0·2	250
S-Methyl hydrochloride	24	25	0·04	625
	26	50	0·04	1250
S-Ethoxycarbonylmethyl	14	10	0·01	1000
2-Mercapto-4-methylglyoxaline				
S-Benzyl	52	50	5	10
	75	100	8	12
S-Methyl	49	50	5	10
Thiourea				
S-Benzyl hydrochloride	27	10	3	3·3
	52	50	6	8·25

(cf. the ready decomposition of *S*-benzylthiourea by alkali giving benzyl mercaptan).

To check this point, however, 2-mercapto-4-methylglyoxaline and 2-benzylthio-4-methylglyoxaline were administered to rats, and the urine was then examined for the presence of the thiol using 2:6-dichloroquinone chloroimide. At pH 8 this reagent gives with 2-mercapto-4-methylglyoxaline a red-brown product, which is extractable with chloroform. The purplish pink solution so obtained can be used for quantitative determination of the thiol with a Spekker photometer by reference to a calibration curve. This technique has been used by McAllister (1951; McAllister & Howells, 1952) for the analysis of 2-mercapto-1-methylglyoxaline and some thiouracils which give yellow chloroform-soluble products with the reagent.

It was found that following administration of 2-mercapto-4-methylglyoxaline at a level of 50 mg./kg. at least 56 % of the dose is excreted in the urine within 18 hr. 2-Benzylthio-4-methylglyoxaline was administered also at a level of 50 mg./kg. The antithyroid effect of this dosage is approximately equivalent to that of 5 mg. of the thiol, and hence one might have expected to find some 2·5–3·0 mg. of thiol in the urine if it were formed from the benzyl derivative. In fact, no thiol was found in the urine, nor when the dosage was increased to 200 mg./kg. In the latter case, a marked odour of benzyl mercaptan was noticed in the urine, which also gave a positive nitroprusside reaction for mercaptan.

A similar experiment was carried out with 2-benzylthio-1-methylglyoxaline hydrochloride (50 mg. of free base/kg.). Again, no thiol was detected in the urine.

Ergotamine and dihydroergotamine

Ergotamine tartrate has been dosed to rats by subcutaneous injection in saline solution, and the effect on radioactive iodine uptake determined in the usual way. Several experiments were carried out, at dose levels of 0·5 and 1·0 mg./kg. body weight.

Radioactive iodine uptakes were markedly depressed, but in some experiments male dosed rats showed much smaller depressions than did the females (e.g., males, 8·2, females, 53·4; males, −5·4, females, 52·8 %). This did not always occur, and in the last experiment with ergotamine recorded in Table 1 eight male rats gave a depression of 29·6 % and ten female rats a depression of 32·8 %. (These values are calculated from control rats of the same sex only.)

Results with ergotamine have been more erratic than with the various antithyroid drug derivatives examined, and the sex difference sometimes found could not be linked with any factor such as the age of the rats or the time of year.

Dihydroergotamine methanesulphonate had no effect on the uptakes of either male or female rats.

SUMMARY

1. A further range of substituted 2-mercaptoglyoxalines has been tested for antithyroid activity in rats.

2. All 4-substituted 2-mercaptoglyoxalines are less active than 2-mercaptoglyoxaline, the most active being the *n*-butyl derivative.

3. *S*-Acyl derivatives of active 2-mercaptoglyoxalines are also active.

4. *S*-Alkyl derivatives of some mercaptoglyoxalines show weak activity.

5. Ergotamine administration reduces thyroidal iodine uptake, a sex difference sometimes being observed.

We wish to thank Mr H. V. Morley for providing a number of the substances examined, Dr R. A. F. Bullerwell for the 2-mercaptoglyoxaline–maleic anhydride adduct, Dr F. Kurzer for the 3:5-diaminothia-2:4-diazole and Dr J. Fernández-Bolaños for the 4-formyl-2-mercaptoglyoxaline. We are indebted to Mr J. O. Stevens for valuable technical assistance, and to Messrs Glaxo Laboratories Research Division who provided most of the rats and diet used. One of us (C.E.S.) thanks the University of London for the award of an I.C.I. Research Fellowship and a grant for the purchase of animal cages.

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The Branched-Chain Fatty Acids of Butterfat

6. FURTHER INVESTIGATIONS ON THE C_{15} SATURATED ACIDS

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In work previously reported (+)-12-methyltetradecanoic and 13-methyltetradecanoic acids were isolated from mutton fat (Hansen, Shorland & Cooke, 1953), and from butterfat (Hansen, Shorland & Cooke, 1954*a*), while *n*-pentadecanoic acid was isolated from mutton fat (Hansen, Shorland & Cooke, 1954*b*). As, however, the isolation procedure in each case involved hydrogenation, it was not established with certainty that these acids were originally present in the fats as saturated constituents.

In this paper it is shown that without hydrogenation, and by use of a highly efficient fractionating column, two fractions were obtained from butterfat, the first of which contained mainly a mixture of the methyl esters of (+)-12-methyltetradecanoic and 13-methyltetradecanoic acids and the second consisted essentially of methyl *n*-pentadecanoate.

EXPERIMENTAL

Optical rotations were measured on the pure liquids without the use of solvents.

Preliminary fractionation. The butterfat used in this work was supplied by the Rangitaiki Plains Dairy Co. Ltd., Whakatane. The crude methyl esters (51 620 g.) prepared from the butterfat were heated on the water bath under the vacuum of a water pump to remove traces of water and solvents which were unavoidably contaminated with a small proportion of the more volatile esters. The residue was distilled in a falling-film still at approximately 2·5 mm. and 135° yielding distillate (V1 + V2, 7323 g) of which the more volatile part (V1, 372 g.) was collected in a dry-ice trap. The residue R1 (44 584 g.) was redistilled in a falling-film still at 130° in high vacuum to give a distillate (V3, 42 453 g.) and a residue (R2, 1841 g.).

V3 (15 889 g.) was distilled *in vacuo* in a Vigreux column (210 × 6 cm.) and the first fraction redistilled in another Vigreux column (480 × 4·5 cm.). From the latter fractionation, fraction 1,6 the saponification equivalent of which approached that of pentadecanoic acid, was selected for the investigation reported in this paper. This concentrate was refractionated in column *G* patterned on column *E* (Shorland, 1952) (cf. Table 1). Column *G* (250 × 3·5 cm. with 3·5 mm. diameter coil) when tested with a 1:1 mixture of heptane–methyl*cyclo*hexane at atmospheric pressure was found to possess more than ninety theoretical plates.

C_{15} *Fractions.* Fractions 1,6,1 to 1,6,3 were not further examined, as they appeared to consist essentially of methyl myristate. The remaining fractions corresponded in saponification equivalent to methyl esters of C_{15} acids. Fractions 1,6,4–1,6,6 which were optically active and possessed low melting points, were combined and denoted A, while fraction 1,6,7 with relatively high melting point and with no detectable optical rotation was denoted B.

Table 1. *Fractional distillation of fraction* 1,6

Fraction	Wt. (g.)	B.p. (*ca.* 0·1 mm.)	Sap. equiv.	Iodine value (Wijs)	M.p. (°)	α_D^{18} (1 dm.)
1,6,1	30·41	93	241·7	23·0	18·0–18·5	Not detectable
1,6,2	29·95	93–94	242·9	3·5	18·5–19·0	Not detectable
1,6,3	19·01	94–98	243·6	2·9	16·8–17·3	Not detectable
1,6,4	31·75	98	258·0	4·2	−10·8 to −8·6	+1·80
1,6,5	34·68	98	257·6	3·1	−13·0 to −11·4	+2·84
1,6,6	11·81	98–99	257·2	11·3	−13·4 to −10·5	+2·84
1,6,7	44·09	—	257·3	5·4	17·8–18·0	Not detectable

Table 2. *Chemical and physical properties of fractions* A 1–5 S, BS *and* CS

Fraction	Wt. (g.)	Sap. equiv.	Iodine value (Wijs)	Refractive index n_D^{60}	M.p. (°)	Combustion analysis (%) C	Combustion analysis (%) H	C-methyl groups	$[\alpha]_D^{18}$	X-ray long spacing (Å)
A 1–5 S	12·76	241·5	0·2	1·4318	52·2	74·3	12·4	1·27	Not detectable	29·7
BS	17·77	242·3	0·2	1·4327	53·3	74·3	12·3	—	Not detectable	35·61
CS	19·63	241·6	0·0	1·4328	24·4	73·7	12·3	1·42	+4·65	30·71 and 27·72
Calc. for $C_{15}H_{30}O_2$:		242·4	—	—	—	74·4	12·5	—	—	—

Fraction A was further resolved in column *G* to give fractions A 1 to A 5 with observed rotations α_D^{18} (1 dm.) ranging from +1·70° to +2·30° and A 6 and AR with observed rotations α_D^{18} (1 dm.) +2·61° and +3·00° respectively.

The combined fractions A 1–A 5 (denoted A 1–5) were converted into acids (38·0 g.) and crystallized 9 times from 40 vol. of light petroleum (b.p. 50–60°) at −40° yielding 12·76 g. of insoluble acids (A 1–5 S, m.p. 52·2°; see Table 2) which, when mixed with an equal amount of 13-methyltetradecanoic acid, supplied through the courtesy of Professor E. Stenhagen, (Arosenius, Ställberg, Stenhagen & Tägtström-Eketorp, 1949), showed no depression in melting point.

Fraction B, which appeared to consist mainly of methyl *n*-pentadecanoate, was purified by fractional distillation in column *G*. Of the resulting fractions those with melting points ranging from 18·0 to 19·1° were combined, converted into acids (20·53 g.) and crystallized 5 times from 40 vol. of light petroleum at −40° giving 17·77 g. insoluble acids (BS) m.p. 53·3° (see Table 2) and 1·98 g. soluble acids (BL).

Soluble fractions A 1–5 as well as A 6, and BL were combined, giving 53·9 g. of acids (denoted C). Fraction C was crystallized from 10 vol. of light petroleum at −40° and the soluble fractions containing unsaturated acids were twice submitted to this process giving each time diminishing proportions of precipitate. The precipitates were combined and crystallized at −40° from light petroleum, using 10 vol., then 30 vol. and finally they were crystallized 4 times using 40 vol., the insoluble fractions being crystallized in each instance. The soluble fractions, except the first, were combined giving 30·77 g. acids. These were recrystallized, first from 10 vol. of light petroleum, and then twice from 40 vol. at −40° giving 19·63 g. of precipitate (denoted CS, m.p. 24·4°; see Table 2) and 11·41 g. soluble fraction.

Further amounts of C_{15} esters were isolated by fractional distillation of V 2 and fractions 1,7 and 1,8 from V 3.

Proportions of C_{15} *acids.* After identification of the C_{15} acids their proportions in the original esters were calculated on the basis that the C_{15} acids were separated sharply from C_{14} and C_{16} acids by ester fractionation (cf. Table 1). Further, the C_{15} acids themselves were separated by this procedure into two groups containing respectively (*a*) mixtures of *iso* and *ante-iso* acids, and (*b*) *normal* acids only. The proportions of *ante-iso* acids present in (*a*) were determined by means of optical rotation assuming that the pure acid had $[\alpha]_D^{18}$ +4·65° and the *iso* acid was determined by difference. In all cases allowance was made for the small proportions of unsaturated constituents which are still under investigation. In this way percentages of C_{15} acids in the total fatty acids of the butterfat were calculated as *normal*, 0·82; *iso*, 0·37, and *ante-iso*, 0·43.

The X-ray long spacings were determined with a Philips Geiger X-ray spectrometer, iron-filtered $K\alpha$ radiation being used. The samples were melted on a glass slide and quickly cooled. Melting points were determined in closed capillaries and are uncorrected.

C, H, and C-methyl analyses were by Drs G. Weiler and F. B. Strauss, Oxford.

DISCUSSION

The analytical data shown in Table 2 demonstrate that fraction A 1–5 S consists of 13-methyltetradecanoic acid and fraction CS consists of (+)-12-methyltetradecanoic acid (cf. Hansen, Shorland & Cooke, 1953, 1954*a*). It should be noted that reliable X-ray long spacings are difficult to obtain for *ante-iso* acids (Velick, 1947).

Fraction BS (cf. Table 2) when admixed with equal proportions of pure *n*-pentadecanoic acid showed no depression in melting point. Further evidence that fraction BS consists of pure *n*-pentadecanoic acid is given by the X-ray long spacing (35·61 Å) as compared with 35·75 Å found by Slagle & Ott (1933) for this acid.

In this laboratory's work on branched-chain fatty acids, the fat has usually been hydrogenated

to convert the unsaturated fatty acids into saturated, and thereby facilitate their subsequent separation from the branched-chain fatty acids by low-temperature crystallization. Under those circumstances it could not be assumed that the branched-chain acids isolated were actually present originally as saturated acids. Moreover, there existed the possibility that hydrogenation might open up a *cyclo*propane ring if present in the original acids (cf. Hofmann, Lucas & Sax, 1952). The present work, therefore, establishes that the *iso*, *ante-iso* and *normal* pentadecanoic acids were present as such in the butterfat, and were not formed from unsaturated or cyclic acids. The isolation, without recourse to hydrogenation, of a C_{20} multibranched fatty acid fraction by distillation and chromatography of the methyl esters of butterfat supports the view that all the branched-chain fatty acids so far isolated from natural fats were present at least in part as saturated constituents.

Saturated straight-chain acids containing an odd number of carbon atoms have not generally been accepted as constituents of natural fats (cf. Hilditch, 1947; Ralston, 1948). However, recent work in this laboratory has led the present authors to abandon this view. Hansen & McInnes (1954) have presented evidence for the occurrence in ox perenephric fat of volatile fatty acids with an odd number of carbon atoms, while Hansen, Shorland & Cooke have isolated *n*-pentadecanoic (1954*b*) and *n*-heptadecanoic acids (1954*c*, *d*) from hydrogenated mutton fat. In addition, unpublished evidence by Morice & Shorland shows the presence of *n*-pentadecanoic acid in shark liver oil. The present work, however, affords the first conclusive evidence that a straight-chain fatty acid with an uneven number of carbon atoms (*n*-pentadecanoic acid) occurs as such in a natural fat. Gray, Pilgrim, Rodda & Weller (1951) have shown by use of isotopic labelling that propionic acid in the rumen is converted into valeric acid, and this result is consistent with our view that saturated acids with an odd number of carbon atoms may well be derived by the successive addition of acetate to propionic acid. It is hoped to test this view experimentally.

The quantity of C_{15} acids isolated amounts to approximately 1·6% of the total fatty acids and does not necessarily represent the total amount present in butterfat, as traces of these acids are likely to occur in other fractions which have not been fully investigated. From the previous papers in this series it is obvious that further investigations now in progress will lead to the isolation of other branched-chain acids and acids with an odd number of carbon atoms and their total quantity will be greater than was hitherto anticipated. Accordingly, earlier results for the fatty acid composition of butterfat, as well as probably those of many other fats, will need to be modified in the light of the present findings.

SUMMARY

1. Two C_{15} branched-chain acids, (+)-12-methyltetradecanoic acid and 13-methyltetradecanoic acid, together with the straight-chain odd-numbered acid *n*-pentadecanoic acid, have been isolated from butterfat by methods which did not involve hydrogenation.

2. Expressed as a percentage of the total fatty acids, the amounts of (+)-12-methyltetradecanoic acid, 13-methyltetradecanoic acid and *n*-pentadecanoic acid were 0·43, 0·37 and 0·82% respectively.

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